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Heated Fats and Allied Compounds as Carcinogens: A Critical Review of Experimental Results [1,2]

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SUMMARY

A critical review of the literature shows no valid experimental evidence that heated fats are carcinogenic on oral administration, and chemical studies have failed to demonstrate known carcinogens in heated fats. From general experience on experimental carcinogenesis in the alimentary tract, it is concluded that feeding experiments on rodents are inadequate for the demonstration of carcinogenic components in human food. Experimental evidence on heated fats as carcinogens after subcutaneous and cutaneous application is critically reviewed, together with results obtained by similar procedures with cholesterol and other unheated lipides. The results are variable and inconclusive. Subcutaneous application is discussed, and it is concluded that this method is not suitable to disclose dietary carcinogens. Thus experimental methods available at present are invalid as evidence on the possible carcinogenicity of heated fats.—J. Nat. Cancer Inst. 25: 893–926, 1960.

THE EVER-INCREASING use of food additives, such as dyes, preservatives, antioxidants, emulsifiers, etc., has markedly increased the importance of the possible presence of carcinogens in food as potential cancer hazards (*1*). There are statistical indications (*2–4*) that human cancer of the gastrointestinal tract proximal to the pylorus may be conditioned by exogenous factors, but detailed demographic studies based on excellent medical systems have failed to demonstrate any increase in cancer of the gastrointestinal tract (*5*, *6*). This result is difficult to reconcile with the presence of new locally active carcinogens in food, even though allowance is made for a reasonable period of induction. The possible carcinogenicity of heated fats, discussed for decades, has again become topical, particularly in relation to the application of various new emulsifiers which are produced by the hardening of lipides. The significance of this problem justifies a systematic and critical review of accumulated evidence, so much the

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more since experimental cancer research has been unable to define clearly the concept of carcinogenicity, while at the same time a number of experimental results call for reassessment.

This review centers mainly on three subjects: 1) chemical changes caused by the heating of fats, with a special view to identification of the presumed carcinogen; 2) feeding experiments made on the possible carcinogenicity of heated fats; 3) experiments made with the use of injections and painting with heated fats.

CHEMICAL CHANGES INTRODUCED BY HEATING OF FATS

This purely chemical subject may be treated briefly. Since cottonseed oil is widely used, *e.g.*, in the manufacture of margarine, Chalmers (*7*) chose this substance for his studies on chemical changes that occur upon heating the oil to various high temperatures. Larsen and Morris (*8*) studied the thermal decomposition of lard and obtained similar results. When the fats were heated, the iodine value decreased with temperature elevation and period of heating, whereas the percentage of unsaponifiable material increased (*7*, *8*). At 300° C., polymerization occurred (*7*), and at 300 to 350° C. polymeric material, decomposition products thereof (present in the unsaponifiable fraction as chemically inert tarry substances), free fatty acids, and ketones were formed. The quantitative proportion of these products varied with physicochemical qualities of the chosen procedure; for instance, decarboxylation was catalyzed by iron. Bis-epoxides have not been demonstrated (*9*, *10*). The results of these chemical studies, however, are not clear and leave us without detailed information on the chemical transformation or its products.

In a study of toxicity and nutritive value, by fractional analyses of several heat-polymerized oils, Crampton *et al.* (*11*, *12*) showed that a large proportion of the di- and polymeric products was not absorbed from the intestinal canal.

The temperature used for cooking usually does not exceed 180 to 200° C. (*13*). Studying the transformation of cottonseed oil when heated under "kitchen conditions," Chalmers (*14*, *15*) found only slight changes in the oil when frying potatoes, and, particularly, no formation of polymeric products. Similarly, Poulsen (*16*) found that the temperature reached when frying roast pork, rissoles, and fried pork was over 250° C. only for a maximum of ½ hour, and only on the surface of the meat. Nevertheless, Peacock (*17*) and Poulsen (*16*) both state that some cooking procedures expose the food to higher surface temperatures. Ivy (*18*) mentions that, in contact with metal, fat may attain a temperature of from 400 to 600° C., "and many kitchens reuse the fat many times, which undoubtedly increases the likelihood of the production of carcinogenic substance."

On the basis of spectrographic studies, a number of authors (*7*, *15*, *19–22*)

agree that heated fats do not contain any known carcinogenic hydrocarbons. The significance of this statement is reduced by Peacock (*17*) who points out that the analytic method would not disclose the presence of "some millions of molecules of a carcinogenic hydrocarbon," a quantity which, at least theoretically, cannot be ruled out as carcinogenic.

The transformation of cholesterol, when heated, will be discussed later in this paper.

APPLICATION BY FEEDING

Heated Fats

For the study of the possible carcinogenicity of heated fats, feeding experiments are the accepted method of choice.

Beck and Peacock (*19*) fed rats an adequate basal diet, with pure lard, cottonseed oil, or a mixed cooking fat (cottonseed oil and whale oil), each heated to 200 to 220° C. for 20 to 30 minutes, on 30 consecutive weekdays. Among 77 rats surviving for more than 4 months, 17 had solitary or multiple papillomas in the forestomach at the time of death (117th–436th day). Hyperplastic changes in the glandular stomach or gastric malignancies were not seen. Controls fed unheated lard or cottonseed oil did not develop papillomas. Similar experiments on mice failed to show significant reaction of the forestomach to heated oils.

Since the experimental rats showed signs of A avitaminosis, Beck and Peacock studied the vitamin-A content of the liver in rats fed heated lard and found it reduced as compared with that of controls. The causal relationship was confirmed in subsequent experiments, and in 1954, Peacock (*10*) reported that heated fats contain a number of relatively unabsorbable, straight-chain hydrocarbons which are good solvents for fat-soluble vitamins and presumably carry them through the intestine. Furthermore, studies by Peacock and Beck (*23*) showed that, unlike rats, mice do not develop vitamin-A deficiency under corresponding conditions.

These experiments demonstrate the importance of the composition of the diet in feeding experiments on carcinogenesis: Changes which might be interpreted as premalignant may sometimes be due merely to dietary insufficiency. For further information, readers may refer to comprehensive tabular surveys of numerous studies from past decades on the relationship between diet and gastric changes, especially in rodents, published by Sugiura (*24*) and Kirby (*25*), which show that a variety of ulcerative and papillary lesions are caused in rodents' stomachs by many types of dietary inadequacy.

Later Peacock and Beck (*9, 21, 26*) induced malignant gastric tumors in a few rodents, by feeding them heated fats, sometimes supported by cocarcinogens.

In the final account of a large experimental series, Peacock, Beck, and Chalmers in 1953 (*9*) reported results with 268 mice fed an adequate diet plus cottonseed oil, heated at 350° C. for 4 hours or heated at 320° C. for 2½ hours in the presence of iron. This fat was administered: suspended in a soap solution, to 167 mice, mixed with dry rat cake to 75 mice, and in a vehicle of 10 or 40 percent alcohol to 26 mice. Of 268 mice surviving for more than 400 days, 18 developed papillomas, 6 had carcinomas of the forestomach, 7 showed adenosis of the pylorus, and 15 had hepatomas.

With alcohol as a vehicle, they found among 26 mice no papillomas, 1 adenosis, and 1 hepatoma, and as pointed out by the authors, this might suggest that the aqueous ether soap solution used as a vehicle had some activity *per se*. This is also indicated by adequate control experiments. However, heated cottonseed oil alone induced 6 papillomas and 2 carcinomas in 63 mice. Incidentally, there were no major differences between the individual groups, and cocarcinogens did not seem to increase the effect. Still, the number of tumors in each group is of the order of magnitude of 0 to 4, so that it is difficult to record variations.

Thus 9 percent of the experimental mice developed tumors of the forestomach, and 5 to 6 percent had hepatomas, while the corresponding values for the total control group were 2 and 1 percent, respectively. Malignant tumors were induced only with heated cottonseed oil.

In the opinion of Peacock and Beck (*26*), the gastric tumors originated from the squamous epithelium of the forestomach or from areas of squamous-cell metaplasia in the glandular stomach. They found a few transitions from benign, branched papilloma to squamous-cell carcinoma, and stressed, without giving any histological details, that the papillomatosis found in these experiments is "quite unlike" the corresponding lesion in vitamin-A deficiency [*see* (*137*)].

Since the great majority of the mainly benign, induced tumors occurred in the forestomach, these experiments were supplemented by an attempt to isolate the effect of heated cottonseed oil on the glandular stomach (*9*). In 78 hamsters the forestomach was removed before the feeding with fat, but the few papillomas that arose were localized to the apparently inevitable stump of the forestomach.

With the same objective, 12 mice fed heated cottonseed oil were given subcutaneous injections 3 times weekly of histamine, which, according to Roca de Vinals (*27*), induces hyperplastic lesions in the forestomach as well as the glandular stomach. This caused considerable damage and degenerative changes in the glandular stomach, and after about 600 days the condition resembled atrophic gastritis. Some mice also exhibited a strange, abnormal glandular proliferation, but definite neoplastic changes could not be demonstrated (*9*).

Several of these large experiments ended with inadequate numbers of animals, and while they have not definitely demonstrated a carcinogenic activity of the heated fat, they do suggest a possible tumor-inducing effect, which on the whole seems fairly indefinable.

Poulsen (*16*), imitating the conditions of ordinary cooking, heated fats

on an open iron plate at a temperature of 275° C. for 30 minutes. A total of 1,029 mice were fed a mixture of standard diet and heated lard with added cholesterol or browned forcemeat with heated lard added. The controls received corresponding untreated foods or merely a standard diet. The experiments were carried through 80 to 116 weeks, most of them for 92 weeks. Apart from some gastric ulcers mainly among old mice in various groups, the result was completely negative, with no malignant tumors of the stomach, and the incidence of 7.4 percent of small papillomas of the forestomach in mice fed highly browned meat balls was not significantly different from the 1.2 percent found among controls.

In spite of a high intercurrent mortality, Poulsen advanced a fairly well-founded conclusion, *viz.*, that the heated fats did not exert a carcinogenic action, whereas a cocarcinogenic action could not be ruled out. It must be pointed out, however, that the heating of fats was not as intense as in most other experiments of this type.

Steiner, Steele, and Koch (*28*) fed 60 mice a basal diet with 5 percent fat from "overfried mixed meats (beef, pork, and suet)," or a 5 percent benzene extract thereof, for 11 to 14 months, but no tumors had occurred in the alimentary tract after 12 to 18 months. The temperature used was not stated, and the daily intake of fat was 145 mg.

Ivy (*18*) saw no changes in 40 rats on adequate diet plus 14 percent fat, including lard. However, when the fat was overheated to 350° C., 12 percent of 41 experimental rats, 12 to 30 months later, showed erosive gastritis, ulcer with or without perforation, and hyperkeratosis with papilloma formation. In experiments with a higher fat diet or a slight dietary insufficiency, preheating of the fat accelerated and increased the incidence and severity of the gastric changes, but among 140 rats no malignant growths appeared during more than 18 months.

Lavik and Baumann (*29, 30*) suggested that the reported carcinogenicity of heated lipides, when fed to rats, might be explained by "an acceleration" of the action of natural carcinogens, analogous to the effect of feeding ordinary fat to mice painted with methylcholanthrene, but finally found only slight differences in accelerating effect between heated and untreated fat. The tumor-promoting effect of fat is presumably due in part to a simultaneous increase in caloric intake.

Heated or Irradiated Cholesterol

Fats with a high cholesterol content.—In a large number of investigations, interest has been focused on cholesterol, especially its transformation products, as the cause of the alleged carcinogenic action of heated fats. Roffo (*31, 32*) conducted comprehensive experiments, using heated fats with a high cholesterol content in the diet of rats (*see* table 1). Similar results were obtained with ultraviolet-irradiated cholesterol (*33, 34*). The malignancy of the induced gastric "adenocarcinomatous" ulcers has been seriously questioned (*35*), and the lesions do not fulfill modern criteria of malignancy in experimental carcinogenesis (*35, 36*).

TABLE 1.—Results of feeding heated fats with a high cholesterol content to rats

Reference	Rats	Strain or type	Sex	Basal diet	Substance (*preparation and dose*)	Animals with tumors*		Survival	Duration of experiments (*months*)	Type of tumor
						In gastro-intestinal tract	In other sites			
Roffo, 1938, 1939 (*31, 32*)	50	Albino	—	Bread and milk	50% Lamb fat | Heated at	Tumors [1]	Tumors [2]	Not reported	26	1. Adenocarcinomas of glandular stomach (average latent period at least 22 months), papillomas of forestomach, adenocarcinoma coeci. Total number of tumors not reported
	50	Albino	—	Bread and milk	50% Suet | 350° C. for					2. Hepatic and pulmonary sarcomas. Total number of tumors not reported
	50	Albino	—	Bread and milk	50% Olive oil | 30 minutes					
	50	Albino	—	Bread and milk	50% Lard					
Lane, Blickenstaff, and Ivy, 1950 (*23*)	202†	From Roffo's colony	—	Bread and milk + vitamins	Lard, heated at 350° C. for 30 minutes	24 [3]	0	144 Alive at 12 months, 64 at 18 months, 12 at 24 months	18–24	3. Papillomas of forestomach
	110†		—		Untreated lard	3 [4]	0	71 Alive at 12 months, 17 at 18 months	—	4. Papillomas of forestomach
Morris, Larsen, and Lippincott, 1943 (*42*)	30	Wistar	—	Adequate	50% Lard, heated at 300° C. for 30 minutes	9 [5]	0	12 Alive at 52 weeks, 5 at 76 weeks	27	5. Papillomas of forestomach
	30		—		35% Lard, heated at 350° C. for 30 minutes + 15% unheated lard			6 Alive at 52 weeks, 2 at 76 weeks		
	30		—		50% Lard, heated at 300° C. for 2 hours			12 Alive at 52 weeks, 1 at 76 weeks		
	20†		—		38% Unheated lard + 12% free fatty acids			12 Alive at 52 weeks, 4 at 76 weeks		

	30†		—		50% Unheated lard			8 Alive at 52 weeks, 3 at 76 weeks		
Morris, 1947 (*13*)	80	—	—	Adequate	35% Lard, heated at 300° C. for 3 hours	0	0	—	12–30	
	24	—	—		35% Unheated lard	0	0	—		
Peacock and Beck, 1943 (*43*)	50	Norwegian hooded	M	Adequate + cod-liver oil	Lard, heated at 350° C. for 1 hour (5–7 gm./rat per week)	0	2 [6]	40 Alive at 372 days	—	6. Multiple mesenteric sarcomas at 372 and 426 days
	36		M		Lard, heated at 220° C. for 12 hours (5–7 gm./rat per week)	0	1 [7]	18 Alive at 363 days	—	7. Multiple mesenteric sarcoma at 363 days
	50		M		Lard, heated at 220° C. for 4 hours (5–7 gm./rat per week)	0	1 [8]	—	—	8. Solitary mesenteric tumor caused by *Cysticercus fasciolaris*
	16†		M		Unheated lard (5–7 gm./rat per week)	0	0	—	—	
	20	Wistar	M	Restricted (bread or potatoes + milk)	Lard, heated at 350° C. for 1 hour	4 [9]	2 [10]	13 Alive at 301 days	—	9. Papillomas of forestomach at 376–432 days 10. One multiple mesenteric sarcoma at 301 days, 1 hepatic sarcoma, caused by *Cysticercus fasciolaris*
	20		M		Lard, heated at 220° C. for 12 hours	2 [11]	1 [12]	—	—	11. Papillomas of forestomach
	8†		M		Unheated lard	0	0	—	—	12. Hepatic sarcoma, caused by *Cysticercus fasciolaris*

*Superior numbers refer to tumors in last column.
†Controls.

Furthermore, criticism has been directed toward the experimental conditions. In Kirby's opinion (*25*), the basal diet used (bread and milk) was probably insufficient in most vitamins, in fat, protein, and possibly mineral salts, as was the standard diet used by Fibiger (*37*). Morris and Lippincott (*38*) state that a deficient ingestion of the food due to the 50 percent fat content may have resulted in secondary inanition manifestations. Moreover, Roffo's theory of oxycholesterol, formed by heating or ultraviolet irradiation of cholesterol as the carcinogenic agent, has been severely criticized by Bergmann (*39*), Veldstra (*40*), and Cook and Kennaway (*41*) as mainly speculative and loosely founded.

Most subsequent workers have been unable to reproduce Roffo's results (*see* table 1). The fat used has been lard. In the well-planned experiment of Lane, Blickenstaff, and Ivy (*22*), only petechial bleedings appeared in the glandular stomach. Since, according to Morris, Larsen, and Lippincott (*42*), Wistar rats can live only for a few weeks on equal parts of an apparently sufficient standard diet and lard heated at 350° C. for ½ hour, these workers modified the dietary regimen as shown in table 1. Despite the rather low survival rate of the experimental rats, it is striking how few gastric changes were induced in this experiment, showing only an infrequent occurrence of small benign papillomas and ulcerative lesions in the stomach.

Peacock and Beck (*43*) obtained abdominal sarcomas in 4 out of 71 rats fed preheated lard (*see* table 1). In this result they saw the action of a potential carcinogen acting upon the mesoblastic tissues along the path of absorption. The low incidence of tumors and the small actual control series, however, do not permit final and far-reaching conclusions.

Cholesterol and cholesterol derivatives.—The experimental evidence given by Roffo (*44*, *45*) and Waterman (*46*) on the carcinogenicity of some cholesterol derivatives after oral administration to rats and mice is not convincing. Roffo found a phenanthrene derivative in a cholesterol fraction obtained by pyrogenic distillation at between 240 and 340° C. Unlike Roffo, Waterman (*47*) believed, with Veldstra (*40*), that the pyrogenic action affects esters of cholesterol, and that dehydrogenation of the latter leads to the formation of cholesta-3,5-diene whose carcinogenic effect he felt was indicated by feeding experiments. Klein and Palmer (*35*) critically reviewed the work of Waterman (*46*), and with other authors (*48–50*) were unable to confirm the alleged carcinogenicity of cholesta-3,5-diene.

Larsen and Barrett (*50*) justly state: "The absence of definite criteria of malignancy in the description of gastric lesions by Roffo and Waterman, the relative ease of induction of keratosis and papillomatosis of the forestomach in rats by a variety of experimental conditions, and the inability of other investigators to induce gastric neoplasia with similar procedures elicit doubt as to the alleged carcinogenicity of heated cholesterol or heated fats administered orally." This statement is supported by Kirby's thorough studies (*25*, *49*, *51*) on the possible carcinogenic action of heated cholesterol fed to rats.

In his first experiments (*25*), Kirby used cholesterol, preheated at 270 to 300° C. for ½ hour, but in an effort to intensify a possible carcinogenic effect, he separated a methanol insoluble fraction of the cholesterol which had been heated at 300° C. (*49*). This eliminated two noncarcinogenic, methanol-soluble products of heating: dicholesteryl ether and cholest-4-en-3-one. Furthermore, cholesta-3,5-diene, formed through intramolecular dehydration of cholesterol, was tested by feeding.

Since cholesterol in food generally forms an ester of one of the higher, fatty acids, and since presumably on oxidation such ester shows a reaction rate different from that of pure cholesterol, Kirby, in succeeding experiments (*51*), fed rats on cholesteryl stearate and cholesteryl palmitate, respectively, heated at 300° C. for ½ hour. Considering that several cooking procedures exceed a temperature of 300° C., he finally used cholesterol, heated at 430° C. for ½ hour.

These various products were fed in a daily individual dose of 20 to 25 mg. to groups of 12 to 24 rats, and most of the animals survived for more than 400 days and up to 730 days. Only three rats developed papillomas in the forestomach, and only to a mild degree. Minor changes like hyperkeratosis and epithelial hyperplasia were seen in approximately 20 percent of the animals, which appear to be common in rats over 18 months of age. The rather frequently observed hemorrhagic erosions in the glandular stomach were explained as a sequela of malnutrition due to purulent lesions in the lungs. Due regard was paid to the possible influence of vitamin-A deficiency.

Kirby failed completely to induce malignant tumors, and although his experimental groups were rather small, this negative result, in spite of considerable observation, strongly militates against the formation of carcinogenic agents after the heating of cholesterol.

Unheated Fats

Domagk (*52*, *see also 35*) reported precancerous lesions and one adenocarcinoma of the glandular stomach in mice fed a high fat diet. The malignancy of the lesions is highly questionable, but the experiments suggest—and this is perhaps their main value—that possibly results like Roffo's may be obtained by a high lipide diet, without heating or irradiation of fats. However, this cannot be decided at present because Roffo did not conduct control experiments with equal parts of basal diet and untreated fat.

Known Carcinogens

To evaluate the reported results, it must be decided whether feeding experiments used as carcinogenic tests have been suited to disclose dietetic carcinogens that may constitute a risk to man. It is relevant, therefore, as positive control, to investigate what has been obtained by feeding rats and mice with known carcinogens.

The results of such investigations are remarkable, because there has been no well-established case of adenocarcinoma in the glandular stomach,

whereas the epithelium of the forestomach has shown fairly marked sensitivity.

Carcinogens in fatty solvents or directly incorporated in the diet.—Collins, Gardner, and Strong (*53*) fed 133 mice of various strains benzo[*a*]pyrene in sesame oil, inducing squamous-cell carcinoma in the forestomach of 22 mice and papilloma of the same site in 69. The carcinomas that Waterman (*54*) induced by benzopyrene in the forestomach of mice are more questionable (*35*).

The most comprehensive and most valuable studies are those of H. L. Stewart and Lorenz, who clearly demonstrated the significance of the vehicle and the varying sensitivity of mouse strains. Methylcholanthrene or dibenz[*a*,*h*]anthracene was administered to mice in aqueous oil emulsions, substituted for drinking water (*55*, *56*). Addition of stabilizing and emulsifying agents showed that the carcinogenic effect on the forestomach depended to a large degree on the acid stability of the emulsion, this quality, together with the wetting power and viscosity, presumably securing an intimate and fairly prolonged contact between hydrocarbon and gastric mucosa.

Under these circumstances, squamous carcinoma of the forestomach was induced in 67 out of 385 mice representing several strains, only a few of which showed sensitivity. Adenocarcinoma of the small intestine was common, the frequency reaching 26 percent in some experiments (*57*, *58*). The incidence of this tumor was largely independent of the strain of mouse, while absorbability of the solvent proved to be of primary importance not only to the induction of this tumor but also to the induction of malignant mesenchymal tumors along the path of absorption. Malignant tumors of the small intestine and the mesentery occurred in a high percentage of experimental mice on direct incorporation of methylcholanthrene in the diet, while this method of application proved ineffective in carcinogenesis in the forestomach (*59*). Papillomas were common in all experiments and in controls drinking mineral-oil emulsion, but no signs of malignancy were observed in the controls, and Stewart and Lorenz (*55*) deny that papilloma should necessarily be a precancerous lesion. Their criteria of malignancy are sober. Carcinoma is diagnosed only in the presence of penetration of all layers of the gastrointestinal wall with formation of a tumor nodule on the serosa (*57*). Occasional downgrowth of atypical glands in the submucosa of the glandular stomach, but with intact tunica muscularis, was interpreted as not definitely neoplastic, since this appearance may also be observed in gastritis (*55*) (*cf.* Roffo's "adenocarcinomas"). Similarly, hyperplastic adenoma-like gastritis with submucosal epithelial downgrowth may be seen in monkeys after exposure to irritants (*60*). The spontaneous adenomatous gastritis of I and DBA mice was not influenced by the administration of hydrocarbon and these very strains were refractory to tumor induction.

In these extensive experiments, comprising a total of 507 mice of various strains, no gastric adenocarcinoma was observed. As Lorenz

and Stewart (*56*) conclude: "It seems reasonable, therefore, to assume that the gastric glandular mucosa of the mouse is refractory to carcinogenesis if the carcinogenic agent (polycyclic hydrocarbon) is contained in the food or drinking fluid." Their experiments indicate that "mice can ingest large amounts of dibenzanthracene and methylcholanthrene, considerably in excess of the amounts necessary for tumor induction by skin painting or subcutaneous injection" (*61*), a fact which seems to reduce the value of the feeding method as a test of weak carcinogens.

Carcinogens not belonging to the polycyclic hydrocarbons have also been unable to induce carcinomas in the glandular stomach of rodents. Mulay *et al.* (*62*) used *p*-dimethylaminobenzene-1-azo-1-naphthalene, while the rats of Druckrey *et al.* (*63*) were fed 4-nitrostilbene. In both experiments the induced gastric tumors were localized in the forestomach.

The combination of dietary insufficiency and oral administration of carcinogen failed to induce tumor formation in the glandular stomach of mice (*64*) or rats (*65*). Even drastic treatment, such as repeated direct alimentary administration of distilled water at 60° C., of 0.25 to 1 N hydrochloric acid, or 0.5 to 1 N sodium hydroxide, combined with the administration of an emulsion of dibenzanthracene, failed to induce adenocarcinoma or precancerosis in the glandular stomach of the mouse (*66*). Similarly, atropine sulfate in the drinking water and repeated subcutaneous injections of this agent or mecholyl chloride exerted no potentiating effect (*66*). However, the 507 mice included in the experiment reached an average age of only 9 to 12 months.

The mucous barrier and the use of lipohydrophilic solvents.—Part of the explanation of the resistance of the glandular stomach to oral carcinogens may be the relatively rapid passage of food here, whereas the forestomach, which makes up about two thirds of the stomach of rats and mice, is more relaxed and by its reservoir function permits a more prolonged contact (*53, 57*). Without doubt, however, more importance should be attached to the so-called mucous barrier of the glandular stomach, consisting of the mucous secretion (*67*), the mucous covering, and the mucous cells of the surface epithelium (*57, 68, 69*). Many attempts have been made to penetrate this barrier:

Oral administration of bile, suggested by Ivy (*68*) as early as 1945, did not increase the effect of methylcholanthrene on the gastric mucosa of the mouse, despite a distinct bile-staining in both parts of the stomach (*70*). The same negative result followed oral administration of a chemical desquamator (*71*) or of an aqueous soap solution as a vehicle for the carcinogens (*67, cf. 9, 26, 35*).

Ivy *et al.* broke the mucous barrier in rabbits by inducing a chronic ulcer in the glandular stomach (*72, 73*), but even so, methylcholanthrene did not prove carcinogenic to the gastric epithelium. However, only 1 rabbit survived for more than 18 months, and the artificially induced ulcers healed within a few months.

According to fluorescence microscopy studies (*74, 75*), benzopyrene in oily solution does not penetrate into the mucosa of the glandular stomach

of the mouse, even after 6 hours' contact (*74*) or after rumenectomy, vagotomy, ligature of the pylorus after a period of starvation, treatment with eugenol or lysozyme, or an increase in the *p*H of the gastric contents up to 5 to 7 (*75*). In contrast, the epithelium of the forestomach showed distinct fluorescence soon after ingestion. Nordén (*75*) found this fluorescence to be most pronounced in the superficial horny layer, from which the benzopyrene permeated to the deeper layers. If the concentration of carcinogen was high, this diffusion persisted for more than 1 day. Ermala *et al.* (*74*) also found persistence of the fluorescence for up to 24 to 48 hours. They obtained essentially the same results in experiments on cats in which the stomach corresponds fairly closely to that of the human, and in mice as well as cats they observed pronounced fluorescence in the walls of the small and large intestine after administration of benzopyrene in oily solution. Thus, these absorption studies show conformity with the location of tumors induced experimentally.

New perspectives were opened up, therefore, at the advent of a number of agents with marked lipophilic as well as hydrophilic properties, *e.g.*, the water-soluble polyethylene glycols ("Carbowaxes") and association colloids. With these substances as vehicles, the fat-soluble carcinogenic hydrocarbons, keeping their carcinogenicity, may be brought into aqueous solution (*76*). Oral administration of such solutions or carcinogens must be expected to eliminate the mucous barrier of the glandular stomach, which is of a highly hydrophilic nature. Studies using fluorescence microscopy (*74–79*) have shown that benzopyrene, dissolved in these synthetic lipohydrophilic vehicles, rapidly and easily permeates the glandular mucosa, predominantly its superficial two thirds (*77*), in mice and cats. If the highly penetrating solvents, such as the polyethylene glycols, are used in nonaqueous form, the benzopyrene will persist in the mucous membrane for up to 6 hours (*74*). In this connection, it should be mentioned that bile salt solutions possess properties that place them among association colloids (*79*).

So far, however, oral administration of carcinogenic hydrocarbons in the lipohydrophilic solvents has given disappointing results, inducing only tumors of the forestomach (*76*). Saxén, Ekwall, and Setälä (*80*, *81*) used weekly tube feeding with 0.04 to 0.12 mg. 7,12-dimethylbenz[*a*]-anthracene in aqueous solutions of various association colloids for a maximum of 6 months. They induced squamous-cell carcinoma in the forestomach of 26 out of 81 mice which survived for 3 to 8 months, whereas another group of 33 showed papillomas. The mucosa of the glandular stomach and the small intestine was entirely refractory to tumor induction, whereas 5 hemangiomas were found in the mesentery of the small intestine or in the spleen. A striking feature is the relatively high incidence of forestomach tumors, after such small doses of carcinogens.

By tube feeding mice once weekly with dimethylbenzanthracene, methylcholanthrene, benzopyrene, or dibenzanthracene, dissolved in a polyethylene glycol, Berenblum and Haran (*82*) also obtained only forestomach tumors, though they used high doses of carcinogen

(1.66 mg. per feeding) on an empty stomach. Their experimental period, however, did not exceed 30 weeks.

Mori *et al.* (*83*, *84*) added 3-methylcholanthrene, in an aqueous polyethylene glycol emulsion, to the drinking water of 235 rats. The daily dose ranged from 0.2 to 5 mg. Of 78 surviving rats, 13 showed adenomatous hyperplasia of the glandular stomach, sometimes occurring after 2 months. This well-marked hyperplasia closely resembled the precancerous lesions described by Stewart *et al.* (*85*). This impression is confirmed by photomicrographs, though the epithelial downgrowth appears to take place in collected formations. Since carcinomas did not occur, the precancerous nature of these changes has not been established.

The negative result of these feeding experiments is probably due to various factors. The latent period of adenocarcinoma in the glandular stomach is undoubtedly considerably longer than the experimental periods. Moreover, in several experiments, the dosage of carcinogen has been too low and the intervals between the administration too long. While lipohydrophilic agents used as vehicles may contribute to the penetration of carcinogen into the mucosa of the glandular stomach, their persistence is rather short, 6 hours at most (*74*, *75*), and Nordén (*75*) could not demonstrate benzopyrene-metabolite fluorescence in the glandular stomach of the mouse like that in the forestomach or in benzopyrene-treated skin. New experiments should be awaited with interest.

APPLICATION OF KNOWN CARCINOGENS DIRECTLY TO STOMACH

Direct application of carcinogenic solutions to exposed glandular mucosa proved ineffective for the induction of tumors in the rat (*86*) and dog (*68*), and the possibility remains that the glandular stomach is insensitive to experimental induction of adenocarcinomas. Certain results (*85*, *87–90*) indicate this assumption may not be correct:

By injecting methylcholanthrene dispersed in horse serum or dissolved in mineral oil into the wall of the glandular stomach, Stewart and Lorenz (*87*, *88*) could induce adenocarcinoma in 11 mice of 3 different strains. Extragastric extension or metastases did not occur, but transplantation was successful in 1 mouse. Among the total of 293 experimental mice treated with methylcholanthrene, benzopyrene, or dibenzanthracene in various vehicles, they found 8 adenoacanthomas, 18 mixed adenocarcinomas and sarcomas, and 30 pure sarcomas. The majority of the malignant tumors occurred in C3H mice. Control experiments would have been desirable, as the injection itself presumably may give rise to tissue destruction, facilitating the epithelial downgrowth (*cf. 91*). It is possible, therefore, that not even Stewart's criterion of malignancy (penetration of all gastric layers with nodes on the serosa) is sufficiently strict for these experiments. The slight biological activity of the induced adenocar-

cinomas may perhaps have been due to the fairly short observation period (less than 1 year), and the one positive transplantation strongly indicates malignancy. The adenomas of the glandular stomach occurring in the experiments, and differing from the adenocarcinomas in not infiltrating all layers, correspond fairly closely, histologically and grossly, to Roffo's "adenocarcinomas" (*cf. 88*, figs. 5 and 6). These results were supported by Stewart, Hare, and Bennett (*85*) who found 23 adenocarcinomas, together with tumors of other types, in 472 mouse stomachs injected with methylcholanthrene.

The same experimental technique applied to rats caused only 4 pure adenocarcinomas in 265 animals of 3 different strains (*89*). The tumors were localized to the juxtapyloric portion of the stomach (*cf. 92*), and 1 invaded the liver, whereas 2 were metastatic. The low incidence of adenocarcinoma indicates that "the rat may prove not to be a satisfactory test animal for the study of experimental adenocarcinoma of the stomach."

Nowell *et al.* (*90*) induced adenocarcinoma of the glandular stomach in 4 mice by neutron irradiation. A total of 162 mice received a single dose (290–580 rep) of whole-body irradiation with fast neutrons and were followed until natural death, up to 21 months after irradiation. The carcinomas were nonmetastasizing, and transplantation was not attempted. Whole-body X irradiation (800 r) yielded only 1 gastric adenocarcinoma among 192 mice. Moreover, neutron irradiation was shown carcinogenic to the cecum and the small intestine in mice.

Spontaneous Incidence of Gastric Tumors in Rodents

In many papers on feeding with carcinogens or agents suspected of carcinogenic action, attempts are made to assess the significance of a relatively small number of induced gastrointestinal carcinomas by a comparison of this result with the low spontaneous incidence of these tumors in mice and rats.

In autopsy material of 142,000 mice (from the Slye colony), all of which had died of "natural" causes and usually at the cancer age, Wells, Slye, and Holmes (*93*) found only 3 adenocarcinomas affecting the pylorus, 8 squamous-cell carcinomas of the cardia, and 3 adenocarcinomas of the large intestine, including 2 localized to a prolapsed rectum. In autopsied rats, too, spontaneous adenocarcinomas of the stomach are extremely rare (*89*).

On the background of these data and of the entire experimental gastric carcinogenesis, Oettlé's studies of 1957 (*94*) marked an epoch. In an autopsy study of 236 Mastomys (*Rattus natalensis*, an African rodent) from the Johannesburg colony, all of which had died of natural causes, Oettlé found carcinoma of the glandular stomach in 98. Unlike experimentally induced glandular stomach carcinoma (*85*, *87–89*), most of the tumors found by Oettlé were undifferentiated, solid carcinomas with minimal mucous secretion, and less than 10 percent showed adenomatous architecture. As a sign of high biological activity, 49 showed metastases,

lymph-borne or blood-borne, and, in one, transplantation was successful, giving rise to symptoms at the end of 7 to 8 months. Apart from 2, all carcinomas were located in the region of the fundus glands (*cf. 92*). Atrophic changes of the glandular stomach were observed, though not outstanding, and intestinal metaplasia was not found. Previously, these conditions have been established hypothetically as endogenous factors in adenocarcinoma formation (*74, 95*). Strangely enough, there were no tumors of the glandular stomach in wild Mastomys, though their age distribution seemed to be the same as that of the laboratory Mastomys.

APPLICATION BY INJECTION AND PAINTING

On the basis of clinical experience it may be assumed that exogenous dietetic carcinogens, if they exist, are weak and require a long latent period (*22*). In experimental work, therefore, many authors have used subcutaneous injection as a quicker test for carcinogenicity of food additives, while others have used skin painting.

Overheated Fats

The most valuable and extensive experiments on subcutaneous injection of overheated fats are summarized in table 2. The two spindle-cell sarcomas induced by Beck (*96*) (table 2, IA) developed close to the remains of the injected oil. Metastases were not observed and transplantation was not attempted. Beck takes it for granted that the two sarcomas were induced by the heated oil, believing that the carcinogenic substance had exerted its action in the course of absorption. It must be admitted that the vicinity to the oil depot might suggest a causal relationship and that the long latent period, as well as the persistence of the oil depot, could be explained by slow absorption of a presumably weak carcinogen. However, the number of experimental animals is so small that this result can at most be suggestive.

In Ivy's experiments (*18*) (table 2, II) the tumor incidence in group A + B did not differ significantly from that in control rats receiving injections of unheated lard (C), whereas untreated controls (D) showed no neoplastic lesions. Ivy's conclusion that the heated lard contained "a low-grade carcinogen" is based on a comparison between the experimental and the untreated control rats, but he admits that results of the control injections do not justify the conclusion that the heating itself has produced a carcinogen.

Steiner, Steele, and Koch (*28*) induced only one tumor in a large series of experiments (table 2, IV A–L). This was a subcutaneous spindle-cell sarcoma in the groin and in contact with "a mass of hardened white fat." In the remaining 19 mice of group C there was local persistence of the injected material for up to 18 months.

TABLE 2.—Injection experiments with overheated fats

Reference		Animal	Strain or type	Sex	Substance (*preparation and dose*)	Site or route	Animals with tumors*	Survival	Duration of experiment (*months*)	Type of tumor
I										
Beck, 1941 (*96*)	A	12 Mice	—	—	Cottonseed oil, 0.5 cc., heated at 340-360° C. for 1 hour	S.C.†	2 [1]	6 Alive at 414 days	—	1. Spindle-cell sarcomas at 414 and 538 days
	B	12 Mice	—	—	Cottonseed oil, 0.5 cc., repeatedly heated at 200-220° C. for a total of 12 hours	S.C.	0	3 Alive at 414 days	—	
	C	12 Control mice	—	—	Unheated cottonseed oil, 0.5 cc.	S.C.	0	10 Alive at 414 days, 6 at 560 days	—	
II										
Ivy, 1955 (*18*)	A	106 Rats	—	—	"Overheated" fat (lard or Spry) 0.1 cc. several times a week for 2–4 weeks	S.C.	6 [2]		14	2. Two benign tumors; 4 malignant
	B	61 Rats	—	—	"Multiple-overheated" fat (overheated for 30 minutes on 5 different occasions) dose as in A	S.C.	6 [3]	All alive at 6 months		3. One benign tumor; 5 malignant
	C	48 Rats	—	—	Unheated fat (lard)	S.C.	2 [4]			4. Two benign tumors
	D	150 Control rats	—	—	No injection	—	0	148 Control rats alive at 18 months		The benign tumors consisted of 1 adenoma, 2 fibroadenomas, and 2 fibromas Malignant tumors consisted of 7 sarcomas, 1 leiomyosarcoma, and 1 carcinoma of the breast. The tumors appeared between 7 and 14 months after first injection
III										
Lane, Blickenstaff, and Ivy, 1950 (*22*)		31 Rats	—	—	Spry, cooked at 270° C.	S.C.	0	31 Alive at 12 months, 19 at 18 months	12–18	

IV Steiner, Steele, and Koch, 1943 (*28*)	A	20 Mice	Own stock of partly inbred albinos	F	Fat from overfried mixed meats (beef, pork, and suet), 5 injections of 0.5 cc. within 4 months	S.C.	0	19 Alive at 12 months, 18 alive at 15 months	30–33	
	B	10 Mice		F	Nonsaponifiable lipides from overfried mixed meats, 100 mg. in 0.5 cc. of sesame oil.	S.C.	0	8 Alive at 12 months, 8 alive at 15 months	27–30	
	C	20 Mice		F	Benzene extract from overfried mixed meats, 5 injections of 0.5 cc. within 4 months	S.C.	1 [5]	18 Alive at 12 months, 16 alive at 15 months	30–33	5. Spindle-cell sarcoma at 424 days
	D	8 Mice		F	Nonsaponifiable lipides of a benzene extract from overfried mixed meats, 100 mg. in 0.5 cc. of sesame oil	S.C.	0	7 Alive at 12 months, 6 alive at 15 months	21–24	
	E	9 Mice	C57BR	M	Nonsaponifiable lipides from the benzene-soluble material of an acetone extract of overfried mixed meats, 150 mg. in 0.5 cc. of sesame oil	S.C.	0	9 Alive at 12 months, 5 alive at 15 months	15–18	
	F	24 Mice 24 Mice		M F	Raw mixed meat extract (nonsaponifiable lipides), 25 mg. in 0.1 cc. of sesame oil	S.C.	0	9 Alive at 12 months, 0 alive at 15 months	12–15	
	G	1 Mice		F	Raw mixed meat extract (nonsaponifiable lipides) 100 mg. in 0.2 cc. of sesame oil	S.C.	0	0 Alive at 12 months, 0 alive at 15 months	9–12	
	H	58 Mice 24 Mice		M F	Nonsaponifiable lipides extract of fried mixed meats, 25 mg. in 0.1 cc. of sesame oil	S.C.	0	1 Alive at 12 months, 0 alive at 15 months	12–15	
	I	36 Mice 12 Mice	Purchased from dealers	M F	Nonsaponifiable lipide extract of baked mixed meats (185°–220° C for 3½ hours), 25 mg. in 0.1 cc. of sesame oil	S.C.	0	1 Alive at 12 months, 0 alive at 15 months	12–15	
	J	26 Mice		F	Nonsaponifiable lipide extract of fried beef, 30 mg. in 0.1 cc. of sesame oil		0	10 Alive at 6 months, 0 alive at 9 months	6–9	
	K	25 Mice		F	Nonsaponifiable lipide extract of fried mixed meats (beef, pork, lamb), 36 mg. in 0.1 cc. of sesame oil	S.C.	0	7 Alive at 6 months, 0 alive at 9 months	6–9	
	L	21 Mice		F	Same as in K, dose 108 mg.	S.C.	0	1 Alive at 12 months, 0 alive at 15 months	12–15	

TABLE 2.—Injection experiments with overheated fats—Continued

Reference		Animal	Strain or type	Sex	Substance (*preparation and dose*)	Site or route	Animals with tumors*	Survival	Duration of experiment (*months*)	Type of tumor
IV—Continued										
Steiner, Steel, and Koch, 1943 (*88*)	M	16 Mice 15 Mice	Own stock of partly inbred albinos	M F	Sesame oil, heated at 350° C., 3 injections of 0.5 cc. within 2 months	S.C.	2 [6] 1 [7]	9 Alive at 12 months, 6 alive at 15 months	21–24	6. Spindle-cell sarcomas at 12 and 20 months 7. Spindle- and mixed-cell sarcoma at 685 days
V										
Burrows, Hieger, and Kennaway, 1936 (*98*)	A	9 Rats 7 Mice	—	—	Petroleum ether extract of lard, heated at 340°–360° C. for 2 hours	S.C.	1 rat [8]	6 4 Alive at 1 year	600 Days, on the average	8. Tumor of fibromatous type with spindle-cell areas, at 608 days
	B	5 Rats 5 Mice	—	—	Alcohol extract of lard, heated at 340°–360° C. for 2 hours	S.C.	1 rat [9]	5 5 Alive at 1 year	600 Days, on the average	9. More cellular spindle-cell tumors at 509 days

*Superior numbers refer to tumors in last column.
†S.C. = subcutaneous.

Thus the carcinogenicity of the fat to the subcutaneous tissue was not proved. Most of the experiments, however, were carried out with non-saponifiable lipide fractions, which, as stated by the authors, possibly did not contain the active principle. Moreover, many of the groups of mice showed but low survival rates.

A more positive result was obtained by subcutaneous application of heated sesame oil. The incidence of tumors in this group (M) is striking, when we consider that none of the 61 mice receiving injections of unheated sesame oil as the vehicle developed sarcoma, though they survived for 1 year or more. Here it is of interest that the heated oil persisted for longer than the untreated oil at the site of injection. However, the decisive experiments included but few mice. Similarly Bischoff (*97*) found sesame oil, which had been aerated at room temperature to produce peroxide, mildly carcinogenic on subcutaneous injection.

A sober histological evaluation is given by Burrows, Hieger, and Kennaway (*98*) (table 2, V). Since the spindle-cell tumors showed no rapid invasive growth, metastases were not observed, and transplantation failed, the tendency to invasion may have been reactive. No tumors occurred in the mice.

In his studies on transformation products of heated cottonseed oil, Chalmers (*15*) injected a number of the products subcutaneously into mice. There was no distinct reaction at the site of injection, though observation was continued for 2 years.

Through a long period, Widmark (*99*) painted the occipital region of 149 male and 92 female mice with alcohol or petroleum extract of (*a*) horse muscle treated at a temperature of 275° C., (*b*) browned butter, and (*c*) roasted coffee. Twenty-six controls were painted with the corresponding unheated products or left untreated. Little change occurred in the skin during the entire application period. Widmark considered absorption by this route to be rather unlikely, whereas oral uptake was expected, since the mice quickly licked off the painted extracts. Changes in the gastrointestinal tract were not mentioned. Whereas none of the male mice showed malignant tumors, 9 mammary adenocarcinomas occurred in 23 female mice surviving for 11 months. The average latent period was 360 days. In the controls, 1 mammary cancer developed within 378 days. Widmark concludes that "extracts of roasted food contain one or more carcinogenic substances." The tumors, however, developed in the female group showing by far the highest spontaneous incidence of cancer, and when this fact is taken into consideration, the number of comparable experimental and control mice seems inadequate.

Cholesterol

Heated, oxidated, or irradiated.—In several experiments pyrolytic cholesterol products have been tested parenterally:

By subcutaneous injection into mice of cholesterol or various cholesterol esters, heated at 270 to 430° C. for ½ hour, Beck, Kirby, and Peacock

(*100*) found 2 spindle-cell sarcomas, distributed in 5 groups among a total of 90 mice, after 345 and 386 days. One was localized to the wall of a cyst containing injected oil. It is not clear whether the remaining mice showed retention cysts at the site of injection. Among 18 mice painted twice weekly with cholesterol, preheated at 430° C. for ½ hour, there were 3 simple, unbranched papillomas appearing at the end of 460 days. At that time, however, the mice had become infected with *Ascarides*. Two papillomas later regressed. The author's conclusion, that heated cholesterol (and its esters) had a weak carcinogenic action, must be regarded with some reserve, since the significance of this low incidence was not tested by control experiments.

The value of Steiner, Steele, and Koch's investigations (*28*) is reduced because of a short observation period, averaging 6 to 9 months. During this period subcutaneously applied cholesterol, preheated at 200 to 300° C. for 2 to 3 hours, failed to induce sarcoma in 50 mice.

According to Roffo (*101*), oxidation of cholesterol at 145 to 150° C. leads to the formation of a tarlike substance. Painting of the ears of 12 rabbits with this cholesterol tar induced a local process of carcinogenesis analogous to that induced by tar itself. At the end of 6 to 14 months all rabbits had tumors. Metastases did not occur. No control experiments were run.

On dehydration of cholesterol during heating at 180° C. for 30 minutes, the hydrocarbon cholesta-3,5-diene is formed, in addition to dicholesteryl ether. Larsen and Barrett (*50*) implanted cholesta-3,5-diene subcutaneously into 30 Wistar rats and 40 C3H mice. About two thirds of the animals were autopsied 13 to 16 months after implantation, but there were no signs of hyperplasia, let alone tumor formation. The same result was obtained by subcutaneous implantation of dicholesteryl ether. Thus, cholesta-3,5-diene proved noncarginogenic also to subcutaneous tissue (*cf. 49*).

Bischoff (*97*) found a number of oxidation products of cholesterol to be carcinogenic when administered with sesame oil as a vehicle. Groups of approximately 30 mice received repeated subcutaneous injections of various compounds and were followed up to 18 months of age. The 3 most potent compounds, 4-cholestene-3,6-dione, 5α, 6α-epoxy-3β-cholestanol, and 6β-hydroperoxy-4-cholesten-3-one, yielded an incidence of local fibrosarcomas ranging from 34 to 66 percent. As an indication of the variability in the results of the subcutaneous test method, 4-cholestene-3, 6-dione in one group of 33 mice induced fibrosarcoma in only 3 percent of the animals, an incidence not significantly different from that of controls. Since the oxidation products that were carcinogenic in sesame oil were noncarcinogenic when administered as aqueous colloids, Bischoff regards sesame oil, which was not found carcinogenic *per se*, as a cocarcinogen. This view was supported by more recent studies (*102*, *103*).

Later Bischoff, Turner, and Bryson (*104*) reported direct carcinogenic action of 5α,6α-epoxy-3β-cholestanol, applied in a noncocarcinogenic vehicle, bovine albumin. In 33 mice, 8 highly malignant fibrosarcomas

appeared at the site of injection. The requirement of a definite concentration of the cholesterol derivative was stressed, low concentrations being ineffective. Hieger (*105*) was unable to reproduce the results of Bischoff *et al.* Of all the oxidative derivatives of cholesterol, he found only 6β-hydroperoxy-4-cholesten-3-one carcinogenic to any appreciable degree. The activity which was tested on C57 mice, while Bischoff used Buffalo mice, was of the same order as that of pure cholesterol.

By subcutaneous injection of cholesterol, irradiated with 2000 to 110,000 r, Burrows and Mayneord (*106*) induced 2 spindle-cell sarcomas among 20 mice at the end of 390 and 544 days, respectively. No tumors were seen in 9 control mice. This material, however, is too small for definite conclusions.

Unheated cholesterol.—While a few investigations suggest that heating, oxidation, or irradiation of cholesterol may lend to it a weak carcinogenic action on connective tissue, the results of Hieger and Orr (*107*) and Hieger (*108*) are surprising. By subcutaneous injection of untreated commercial cholesterol dissolved in lard, Hieger and Orr induced 17 local spindle-cell sarcomas among 193 mice of various strains. One hundred and fourteen were alive at the end of 1 year, 18 at the end of 2 years, and the latent period of the tumors ranged from 11 to 29 months. Comparing results obtained with commercial cholesterol and with highly purified cholesterol, both dissolved in olive oil and yielding a tumor incidence in mice of 3.6 and 4 percent, respectively, Hieger and Orr are inclined to think that cholesterol is carcinogenic in itself.

This theory received further support in Hieger's study in 1957 (*108*). He gave 224 mice subcutaneous injections of highly purified cholesterol in olive oil. Eight and a half to 23 months later, 14 mice developed local sarcomas. These experiments were performed on a stock of mice that had previously (*107*) shown greater susceptibility than C57 mice to induction of sarcoma by cholesterol. Seven hundred controls were injected with olive oil or lard, but only 2 sarcomas were observed in 2 years. Cholesterol suspended in 4 percent gelatin resulted in no tumors, and tumors were induced only when mice were given cholesterol in oily solutions. The experimental technique included 3 to 5 injections of the sterol solutions with the object of maintaining the small subcutaneous depot, whereas, Beck, Kirby, and Peacock (*100*) gave only one injection of heated cholesterol.

In summing up, Hieger (*109*) reports an over-all incidence of 70 sarcomas in 1,434 mice after injection of oily solutions of cholesterol, but the number of tumors varied considerably between groups of animals. Among mice receiving injections of pure cholesterol, the percent of sarcomas calculated on survivors at 12 months varied between 0 and 14. Hieger finds no obvious alternative to the explanation that the fluctuations in response are due to susceptibility differences of congenital or environmental origin. It is suggested that such variations of sensitivity are revealed by carcinogenic stimuli of low intensity (*105*).

Bischoff (*97*) found purified cholesterol noncarcinogenic on subcu-

taneous injection into mice, and Hieger and Orr (*107*) reported that neither Truhaut (*110*) nor Steiner (*111*) could demonstrate a carcinogenic action of purified cholesterol and therefore believe that commercial cholesterol contains highly carcinogenic contaminants. However, their results do not seem to convince Hieger and Orr. Falk, Goldfein, and Steiner (*112*), Fieser (*113*), and Kennaway (*114*) are inclined to believe that possibly the carcinogenic action is due to a conversion product of cholesterol. Falk *et al.* think that a carcinogen is formed from cholesterol before contact with the organism, and that heat is the most likely agent to cause this change. Hieger's arguments against this theory are not convincing. On pyrolysis of cholesterol at 360° C., a temperature which causes complete decomposition of cholesterol, Falk *et al.* separated a number of intermediate links in the transformation of cholesterol to methylcholanthrene. Neither this nor other known carcinogens were, however, demonstrated. Among the pyrolysis products were six of unknown or uncertain carcinogenic activity.

Moderately Heated Fats

As apparent from the experiments reviewed, interest has focused mainly on intensely heated fats, perhaps inspired partly by Kennaway and Sampson's production of a carcinogenic, tarlike fluid by distillation of cholesterol in a tube at a temperature of 800 to 810° C. (*115*). However, it is still possible that relatively low temperatures may also exert an influence on lipides and lead to carcinogenicity. This has been studied in a number of control experiments from studies on hydrocarbon carcinogenesis, because the solution of lipophilic hydrocarbons in oil usually will require a moderate heating. A few experiments of this type will be reviewed:

Among 31 chickens receiving repeated injections, into the right pectoral muscle, of dibenz[*a*,*h*]anthracene dissolved in lard heated at 140° C. for ½ hour and, into the left pectoral muscle, of heated lard alone, Peacock (*116*) found 7 spindle-cell sarcomas on the right side and 3 sarcomas on both sides. The unexpected development of tumors on the left side might perhaps be due to local release of remote effects of the dibenzanthracene absorbed from the injection site, but Peacock thought it more likely that the experiment indicated a carcinogenic effect of preheated lard, and thus entered into his yearlong studies of the role of heated fats in carcinogenesis.

Andervont's 190 control mice received 3 subcutaneous injections of lard preheated to 140° C., but none of the mice developed tumors. The observation period was 27 weeks, sufficient for the development of dibenzanthracene-induced spindle-cell sarcomas, but no doubt too short to disclose a weak carcinogen (*117*).

In Hieger's control experiments (*105*), various fatty solvents (lard, olive oil, stearic acid plus olive oil, tristearin plus olive oil, and sesame

oil) preheated at water-bath temperature, were injected into mice. Five sarcomas developed in a total of 1,122 treated mice.

Ten groups of 91 rats were included in the control experiments of Walpole *et al.* (*118*) testing arachis oil, presumably sterilized as in the main experiments, by heating at 140° C. for 1 hour. Subcutaneous injections were administered several times a week for 33 to 300 days. In 14 rats sarcoma appeared at the site of injection at the end of 421 to 694 days. Thirteen of these tumors were observed among 48 rats that had been subjected to the treatment for 97 to 300 days. Only a few showed metastatic activity, but most tumors invaded skin or muscles. "To what extent these miscellaneous tumors may be attributed to the oil is uncertain." In contrast to this result, the subcutaneous tissue of C3Hf and C mice proved resistant to tumor induction by arachis oil despite an observation period of up to 504 days.

Unheated Fats

A few of the findings might indicate a certain sarcomagenic action of lightly heated fats, at any rate in the more sensitive strains of rats. The heating itself, however, need not be the cause, as shown by several comprehensive control experiments with untreated lipides (*119*).

Burrows, Hieger, and Kennaway (*98*) injected subcutaneously various fats, oils, fatty acids, or liquid paraffin into 236 mice, but an experimental period of an average of 443 days did not show any tumors at the site of injection. In 193 rats, subcutaneous depots of lard or olive oil, in some with the addition of various substances, were maintained through an experimental period averaging 88 weeks. Among 99 rats surviving for 1 year or longer, there were 8 spindle-cell tumors, presumably of low malignancy, at the site of injection.

Since these tumors showed invasive growth and caused the death of the host, but did not metastasize, nor did they take a transplantation, their malignancy was qualified and corresponded in aggressiveness to human fibromatosis. The incidence of tumors was considerably lower than with dibenzanthracene, and the latent period materially longer. After repeated intraperitoneal injections of olive oil or lard, 1 rat of 60 and 2 mice of 80 developed multiple, lymphosarcomatous peritoneal tumors at the end of 27 to 62 weeks. Burrows *et al.*, however, did not feel justified in establishing whether the tumors originated from the peritoneum or were due to the injections.

Barry and Cook (*123*) have reported "a certain amount of neoplastic reaction" in 2 out of 10 control rats receiving subcutaneous injections of lard. Autopsies were carried out on the 385th and 462d days, and the tumor-like tissue consisted mainly of necrotic and granulation tissue. The suspected areas were made up of spindle cells, but were far less dense than in hydrocarbon tumors. The tissue "showed none of the characteristics of malignancy," there were no metastases, and trans-

plantation experiments were negative. A similar experiment on 20 mice did not lead to lesions of this nature.

After repeated subcutaneous injection of pure sunflower oil into 16 mice surviving for a maximum of 6¼ months, Schabad (*120*) observed local oleomas, but does not mention actual tumors. Within the same period, 5 experimental mice receiving injections of dibenzanthracene in sunflower oil developed polymorphocellular sarcoma.

Lastly, Domagk (*52*) mentions that in several of an unspecified number of mice given frequent intramuscular injections of olive oil, he found peculiar oil cysts and a few genuine sarcomas.

Epoxides

Upon oxidative polymerization of oils, epoxides may be formed. A number of these substances with epoxy groups have shown cytotoxic and tumor-inhibiting effect and have therefore been suspected of carcinogenicity. Bis-epoxides, in particular, have been the subject of several injection and painting experiments (*121, 122*).

Twenty-eight rats received several intraperitoneal injections of 1,3-butadiene dioxide or 4-vinyl cyclohexenedioxide, dissolved in peanut oil (*121*). Each substance induced one metastasizing "mixed-cell" sarcoma, one of which proved transplantable. The latent periods were 13 and 7 months. It is difficult to assess the significance of the 2 sarcomas induced by the injection, especially since no controls were run. Painting of mice with butadiene dioxide, dissolved in peanut oil or acetone, proved negative, and the preliminary carcinogenicity of vinyl cyclohexenedioxide (*121*) was disproved in a later painting experiment, with a purer sample of the diepoxide (*118*).

Thorough testing of 2 epoxy resins was made by Hine *et al.* (*122*). Both substances contained 2 epoxy groups: A, aliphatic and water soluble, and B, aromatic and very slightly soluble. Groups of 30 C3H male mice were painted on the skin for up to 24 months with epoxide A, epoxide B, methylcholanthrene as a positive control and the solvent acetone as a negative control. The application of epoxide A induced a few, local carcinomas or sarcomas, the number seemingly dependent on the strength of solution and the frequency of painting. Epoxide B gave negative results as did painting with acetone. All 20 mice that survived 6 months' painting with methylcholanthrene showed tumors at the site of application: squamous-cell carcinoma in 16, subcutaneous fibrosarcoma in 3, and papilloma in 1.

Sixteen male rabbits were exposed to the same procedure, and 13 survived the experimental period of 2 years. Acetone and the 2 epoxy resins did not induce any tumors, whereas painting with methylcholanthrene gave rise to squamous-cell carcinoma in 14.

Injection of epoxy resin A 3 times into the occipital region of 30 male rats gave rise to local fibromas in 2 and sarcomas or fibrosarcomas in 8, as well as 1 unidentifiable tumor stated to be malignant, after an average

experimental period of 402 days. Administered in the same way and with the same average latent period, epoxy resin B induced 4 fibrosarcomas in 30 rats. Negative control experiments with the vehicle propylene glycol did not give rise to tumors, whereas dibenzanthracene, within an average period of 337 days, induced malignant tumors at the site of injection in 17 out of 30 rats.

Intraperitoneal and subcutaneous injection of the two resins or of other epoxides, including epoxidized soybean oil did not, unlike methylcholanthrene, alter the incidence of pulmonary adenomas in male A/He mice in the course of 16 weeks. The same negative result was seen in feeding with the resins for 11 months, and such oral administration through 16 weeks had no influence on the incidence of methylcholanthrene-induced pulmonary adenomas in the mice.

Hine and associates rightly conclude that the carcinogenicity, if any, of these epoxy resins is presumably not very high. Painting of the fairly resistant rabbits proved negative, and the various epoxides were unable to raise the incidence of pulmonary adenomas in strain A mice. The assumption of a low oncogenicity is not contradicted by the positive sarcomagenic experiments, since rats are highly sensitive to subcutaneous implantations.

DISCUSSION

The object of this review has been to illustrate the role of heated fats in carcinogenesis. The practical conclusion to be drawn from the experimental results must be decisively influenced by the value of the applied methods as tests for carcinogenicity of dietary components.

Any injection into the connective tissue of an animal is tantamount to a trauma. After this initial trauma, an inoculum remains which disturbs the tissue equilibrium in countless ways, inducing both physical and chemical reactions of varying severity, *e.g.*, necrosis (*124*). The significance of these factors to the value of the injection method as a test of carcinogenicity must not be underestimated. This is perhaps most distinctly apparent from Takizawa's study (*125*) demonstrating in mice a sarcomagenic action of glucose, fructose, and galactose in hypertonic (25%) solutions injected subcutaneously daily or every other day.

Similar experiments have been performed on rats by Nishiyama (*126*) who induced sarcoma by a highly concentrated solution of glucose in 23.8 percent of rats that survived for longer than 200 days. Moreover, Tokoro (*127*) obtained 1 reticulosarcoma and 1 spindle-cell sarcoma among an unspecified number of albino rats receiving subcutaneous injections of a 15 percent saline solution daily, or every other, or 3d day.

The marked influence of nonspecific factors on subcutaneous sarcomagenesis is also apparent from Peacock's demonstration (*128*) of the significance of the vehicle. Injection of dibenzanthracene dissolved in

chicken fat did not lead to tumor formation in fowls, presumably because of the inability of homologous fat to induce local, proliferative connective-tissue reaction (*124*). When we consider also that cholesterol has been able to induce sarcomas in mice, possibly because of transformation in subcutaneous environment, and that a few tumors or tumor-like masses have arisen after injection of ordinary untreated fats, it must be clear that subcutaneous application differs essentially from oral administration. Similar views have been advanced by Lehman and Nelson (*129*) and Hine *et al.* (*122*), while Boyland (*130*) prefers parenteral injection as a test of the carcinogenicity of food additives. Adherents of the latter method must realize, however, that carcinogens thus defined, *e.g.*, cholesterol or glucose, are unavoidable components of food.

As to heated fats, the well-planned and comprehensive feeding experiments from Peacock's laboratory are, so far, unique in inducing definite gastric malignancies. However, the carcinomas were few and all of them indubitably derived from the forestomach, so that it is difficult to interpret these results and impossible to extrapolate them to the glandular stomach.

Referring to experiments with known carcinogens, we may conclude that carcinogenicity of a suspected agent fed to mice and rats will result in the appearance of tumors in the forestomach, small intestine, and/or mesenteric tissues. Malignant tumors of the liver have not been included in these studies on oral administration of carcinogenic hydrocarbons and will be expected when the carcinogenicity of dyes is tested. Nor have tumors of the large intestine been induced by feeding with carcinogenic hydrocarbons. Experiments have shown that in this part of the alimentary canal, the active hydrocarbon has been converted into a noncarcinogenic compound, perhaps by the intestinal flora (*56*). Strangely enough, induction of tumors in the small intestine has been successful only with Stewart and his colleagues. The positive result obtained by those workers may, however, be explained by the use of high doses and particularly sensitive strains of mice. Moreover, a thorough autopsy technique may have contributed to the high incidence. Incidentally, the digestibility and absorbability of the vehicle are of decisive significance to the appearance of carcinoma in the small intestine and of the rather few mesenchymal mesenteric tumors (*56*). This leaves the benign and malignant forestomach tumors as the most constant and most easily reproducible results of oral administration of carcinogenic hydrocarbons.

The appearance of forestomach papillomas cannot, however, be accepted even as a faint indicator of carcinogenicity of an agent tested by mouth, because the mucous membrane of the forestomach, particularly in the rat, is very labile, responding to a number of different actions by the formation of ulcers, diffuse or more localized hyperplasia, and papillomas. Such changes may be induced by mechanical irritation (*24*, *35*) or by faulty nutrition of varying nature, *e.g.*, vitamin deficiency, or lack of amino acid (*24*, *35*, *37*, *131–136*). Similar effects are caused by restriction of the daily intake, or total or intermittent inanition (*35*, *38*). Peacock and Kirby's distinction (*137*) between gastropapillomatosis associated with

dietary insufficiency and the papillomas with some invasive property induced by carcinogenic hydrocarbons seems inadequate, when we consider the often reported epithelial downgrowth of papillomas in nutritionally deficient rats (*35*, *38*, *131*, *136*). The influence of nutrition must be considered an important source of error in feeding tests with carcinogens, because some factors are unknown and still others difficult to control (*131*, *135*, *138*).

It seems beyond doubt, therefore, that the carcinogenicity of an agent to the forestomach can be established only if it is able to induce malignant tumors at this site. If this is true, there are still enormous difficulties in interpreting the result with a view to the risk in the human being who does not possess an analogue to the forestomach of rodents.

It has been pointed out by Klein and Palmer (*35*) that a sharp distinction must be made between squamous-cell carcinoma of the forestomach and adenocarcinoma of the glandular stomach, and that the former must be related rather to esophageal and cutaneous carcinoma.

Barrett (*69*) found it more logical to collate tumors of the forestomach to corresponding changes in the mouth, pharynx, and esophagus of man than to adenocarcinoma of the stomach. An indication of the relationship between tumors of the forestomach and of the skin is found in Collins, Gardner, and Strong's investigations (*53*) which showed a parallel variation in the sensitivity of mouse strains with respect to forming benzopyrene-induced tumors in the forestomach and skin, and an inverse proportionality between the degree and malignancy of the changes in the two sites.

It seems inevitable to admit that the differences between the human and the rodent stomach, anatomically, physiologically, and pathologically, will rule out any comparison in respect to carcinogenesis by the oral route, so that the ordinary test animals, mice and rats, are not suitable for demonstrating carcinogenic components of human food.

If we consider again the question of the carcinogenicity of heated fats, it seems that, so far, the slight suspicion of such effect aroused by the investigations of Peacock and associates has not proved justified in man. Peacock (*17*) has admitted that feeding experiments have given less reason to assume the existence of a carcinogenic action than have injection experiments. In his opinion, however, this does not exclude the possibility that heated fats may be factors in the formation of gastric malignancy. He suggests that small, even nondetectable quantities of carcinogens in the diet, if supported by cocarcinogens, may in time induce tumors. However, subsequent feeding experiments have, so far, (*9*, *26*) failed to support this theory. The few malignant tumors induced by feeding experiments are rather varied, and, as might be expected, most of them are squamous-cell carcinomas of the forestomach, whose significance has been discussed. There was no instance of carcinoma in the small intestine, which might no doubt be interpreted as relevant information, and sarcomas occurred as an isolated phenomenon in one single experiment (*43*).

It seems difficult to escape the conclusion that experiments on car-

cinogenesis with methods available at present are invalid as evidence of the possible carcinogenicity of heated fats.

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Reduction of Transplantability of Novikoff Hepatoma Cells Grown *In Vitro* and the Consequent Protecting Effect to the Host Against Their Malignant Progenitor [1, 2]

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SUMMARY

In 3 lines of the Novikoff rat hepatoma grown *in vitro*, the chromosome complement of the tumor cells showed drastic changes and the cells lost their transplantability. Susceptible animals, after receiving several inoculations of cells grown *in vitro*, became partially immune to the challenges of malignant Novikoff cells carried *in vivo*. It was proved that the cells inoculated as "antigen" must be *viable but nonproliferative*. Attempts to use as antigen the malignant cells *in vivo* irradiated at a dose which allowed survival, but no growth of tumors, also succeeded in protecting the hosts.—J. Nat. Cancer Inst. 25: 927–935, 1960.

IN PREVIOUS communications from this laboratory, it was reported that when Novikoff hepatoma cells were cultivated *in vitro* for a considerable period, their chromosomal modality gradually shifted from single stemline number to double-stemline number, and later to a zone of heteroploid numbers (*1*, *2*). Accompanying the cytological transformation was a loss of transplantability. Moreover, susceptible animals receiving several inoculations of the transformed cells grown *in vitro* became partially immune to challenge with malignant Novikoff implants carried *in vivo* (*3*). It was subsequently noted that the loss of transplantability of the cells *in vitro* was not absolute because they were able to develop into tumors to a limited degree if injected intramuscularly into the host. Subcutaneous inoculation and, from our limited observations, intraperitoneal inoculation of cells *in vitro* invariably failed. Cultivation *in vitro*, therefore, seemed to be a means of attenuating the tumor cells. The present report summarizes results of transplantation experiments made between 1957 and 1959.

Since neither the original rat strain from which the Novikoff hepatoma was induced nor the rat strain used in this study was inbred, it should

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be emphasized in the beginning that the experiments reported here deal with problems pertaining to homotransplantation.

MATERIALS AND METHODS

The animals used for experimentation were all male Sprague-Dawley rats. Unless otherwise stated, they weighed approximately 120 to 140 gm. when the experiments started. They might, of course, reach over 400 gm. in experiments of long duration.

The stock tumor was carried intraperitoneally in susceptible rats. Generally 1 ml. of thickly suspended tumor cells was used to transplant the stock tumor, and the animals succumbed between 6 and 10 days after implantation.

After preliminary testing to prove that all 3 lines *in vitro* of the Novikoff tumor—N-1, N-3, and N-4—behaved similarly in transplantation experiments, strain N-1 cells were used in all experiments reported in this article.

Methods for carrying strain N-1 were the same as described previously (*2*). When inoculations were made, both with cells *in vitro* and cells *in vivo*, cell counts were made at first without discriminating on the basis of viability. In later stages, only viable cells were counted [for methods, *cf.* (*4*)].

Inoculation of cells *in vitro* for tumor development was made via subcutaneous and intramuscular routes. For all other experiments only the subcutaneous route was chosen. Challenge with cells *in vivo* was invariably made intraperitoneally. All control animals were the same age as the experimental sets. They were always purchased at the same time before experiments started. After challenge, the animals were observed for 30 days. If they remained healthy, they were considered resistant and the experiment was concluded.

RESULTS

Reduction of Transplantability of N-1 Cells

From many observations on the transplantability of the N-1 cells injected subcutaneously, we concluded that the cells carried *in vitro* failed to grow in the host susceptible to the Novikoff hepatoma, the progenitor of the N-1 line. Later, a few transplants made intramuscularly proved that the cells could grow. A series of experiments compared the transplantability and the rate of tumor growth of Novikoff cells *in vivo* and N-1 cells. Two routes of transplantation, *viz.*, subcutaneous and intramuscular, and 3 host sizes, 50 to 60 gm., 120 to 140 gm., and 180 gm., were used.

Table 1 summarizes the data obtained. The malignant tumor cells took in both routes and grew equally well. The experiment was ter-

TABLE 1.—Tumor takes and tumor weights (in gm.) after 2 routes of inoculation with Novikoff cells and N-1 cells (inoculum 5×10^6 cells per rat)*

Weeks after inoculation:			1		2		3		4		6		8	
Cell type	Body weight of host (*gm.*)	Route of inoculation	Take	Average tumor weight	Take	Average tumor weight	Take	Average tumor weight	Take	Average tumor weight	Take	Average tumor weight	Take	Average tumor weight
Novikoff *in vivo*	120–140	Subcutaneous	5/5	4. 4	5/5	11. 6								
		Intramuscular	5/5	7. 4	4/5	11. 7								
N-1 cells *in vitro*	50–60	Subcutaneous	0/5	0. 0	0/5	0. 0	0/5	0. 0						
		Intramuscular			0/5	0. 0	0/5	0. 0	0/5	0. 0	0/5	0. 0	1/5	0. 5
	120–140	Subcutaneous			0/5	0. 0			0/5	0. 0				
		Intramuscular	0/5	0. 0	0/5	0. 0	0/5	0. 0	2/5	2. 0	5/5	2. 2	2/5	11. 7
	180+	Subcutaneous	0/5	0. 0	0/5	0. 0								
		Intramuscular									0/5	0. 0	0/5	0. 0

*Each experiment consisted of 5 rats autopsied separately. Weight of tumor average value from tumor-bearing animals only.

minated after the 2d week because most of the hosts died after this period. The N-1 cells, however, did not develop in animals in any weight class when inoculation was made subcutaneously. When transplanted intramuscularly, tumors could not be detected, at least by gross inspection, during the first 3 weeks. Beginning the 4th week, tumors that grew could be dissected and weighed. It can be seen that the average N-1 tumor weight after 8 weeks of incubation was similar to that of the Novikoff tumor after 2 weeks. The difference in takes between the 2 weight classes after intramuscular inoculation was probably due to chance because additional experiments with rats of 50 to 60 gm. body weight showed higher takes than the data indicated in table 1.

Protection Capacity of N-1 Cells Against Tumors *In Vivo*

Table 2 summarizes the data obtained from experiments with N-1 cells as the protecting agent. Each rat received 3 subcutaneous inoculations of N-1 cells (4×10^6 cells per inoculum) at various intervals. Challenge with Novikoff cells *in vivo* was also made at various intervals. The results clearly show that inoculations with N-1 cells offered some protection to the hosts against the implantation of malignant tumors. Table 2 also indicates that a 1-week inoculation interval was insufficient for stimulating host resistance. Combining all experiments, the total value was still significant enough to demonstrate the effect of immunization with "attenuated" tumor cells.

TABLE 2.—Protection of rats from Novikoff hepatoma by subcutaneous inoculations of N-1 cells (4×10^6 cells per inoculum) with used medium as agent† for suspending cells—challenges made intraperitoneally

Procedure of inoculation*	Challenge dose ($\times$ *10^6 cells*)	Mortality of hosts	
		Experimental	Control
0:1:1—1	5	3/5	5/5
0:1:1—2	5	3/5	5/5
0:1:2—2	5	0/5	5/5
0:1:2—3	5	0/5	5/5
0:2:2—2	10	1/5	4/5
0:2:2—3	10	1/5	5/5
0:2:2—2	10	2/5	4/5
0:2:2—3	10	1/5	4/5
0:3:3—3	10	0/5	4/5
0:3:3—4	10	1/5	4/5
0:4:4—3	10	3/5	4/4
0:4:4—4	10	2/5	4/4
0:4:1—1	5	0/2	1/2
0:3:3—2†	10	2/5	2/4
0:3:3—3†	10	1/5	4/5
Total		19/72	60/69

*"0" = initial inoculation; figure after colon = number of weeks between previous inoculation and the inoculation in question; figure after dash = number of weeks after last inoculation when challenge was made.

†Inoculum size 1×10^6 cells.

Treatments to the Antigens

The N-1 cells used for inoculation in the experiments listed in table 2 were all taken directly from culture vessels. The cells were viable when they were injected into the hosts. Table 3 lists the results of experiments designed to detect whether viable antigen was necessary for protecting the hosts. As expected, washing the cells with Gey's balanced salt solution or trypsinization did not seriously impair the effectiveness of the antigen; but methods of killing the cells, including simple freezing and thawing, fixation, homogenization, and heating to 56° C., resulted in a loss of protection to inoculated animals.

TABLE 3.—Protection of rats from Novikoff hepatoma by subcutaneous inoculations of N-1 cells after various treatments (4×10^6 cells per inoculum)—challenges made intraperitoneally

Procedure of inoculation*	Treatments to antigens	Challenge dose ($\times 10^6$ cells)	Mortality of hosts	
			Experimental	Control
0 : 3 : 3—2	Trypsinized	10	2/5	5/5
0 : 3 : 3—3	Trypsinized	10	1/5	5/5
0 : 3 : 3—3	Gey's solution washed	10	2/5	5/5
0 : 3 : 3—4	Gey's solution washed	10	2/5	5/5
Total viable cell "vaccine"			7/20 (35%)	20/20 (100%)
0 : 3 : 3—3	Fixed with 10% formalin	10	5/5	5/5
0 : 3 : 3—4	Fixed with 10% formalin	10	4/5	3/5
0 : 2 : 2—2	Fixed with 1% formalin	2	4/5	3/5
0 : 2 : 2—2	Homogenized	2	3/5	3/5
0 : 2 : 2—2	Heated to 56° C., 30 minutes	2	5/5	5/5
0 : 2 : 2—3	Heated to 56° C., 30 minutes	2	4/4	5/5
0 : 3 : 3—2	Frozen and thawed	10	4/5	5/5
0 : 3 : 3—3	Frozen and thawed	10	5/5	4/5
Total killed cell "vaccine"			34/39 (87%)	33/40 (83%)

*"0" = initial inoculation; figure after colon = number of weeks between previous inoculation and the inoculation in question; figure after dash = number of weeks after last inoculation when challenge was made.

Other Material as Antigens

Since the N-1 cells were suspended in their original culture medium (table 1), it was necessary to be certain the medium was not antigenic. Also of interest was whether normal and viable rat tissues, such as liver, could induce protection against the Novikoff tumor as well. A heterologous cell line (strain L of the mouse) was also used in one experiment. All were negative (table 4).

Lack of Demonstrable Antibodies

Resistant rats were bled 2 or 3 weeks after a booster inoculation of N-1 cells. Pooled serum was mixed with growth medium in various proportions, from 5 to 50 percent. Serum from normal, nonimmunized rats was used as control. Cells of the N-1 strain were seeded in T-15 flasks

TABLE 4.—Protection of rats from Novikoff hepatoma by subcutaneous inoculations of agents other than N-1 cells (4–5 × 10^6 cells per inoculum)—challenges made intraperitoneally

Procedure of inoculation*	Antigen	Challenge dose (× 10^6 cells)	Mortality of hosts	
			Experimental	Control
0 : 2 : 2—3	Rat liver	10	5/5	3/5
0 : 2 : 2—4	Rat liver	10	3/4	5/5
0 : 2 : 2—3	Rat embryo	10	4/4	5/5
0 : 2 : 2—4	Rat embryo	10	5/5	4/5
0 : 2 : 2—3	Rat spleen	5	5/5	5/5
0 : 2 : 2—2	Used medium	5	9/10	10/10
	Fresh medium	5	8/9	
0 : 2 : 2—3	Used medium	5	7/10	7/10
	Fresh medium	5	8/9	
0 : 2 : 2—2	L cells (mouse)	2	5/5	5/5
0 : 2 : 2—3	L cells (mouse)	2	5/5	5/5

*"0" = initial inoculation; figure after colon = number of weeks between previous inoculation and the inoculation in question; figure after dash = number of weeks after last inoculation when challenge was made.

with 2 ml. medium in each vessel. No inhibition of growth or cellular damage was recorded for any rat. Addition of complement (guinea-pig serum) to the growth medium did not change the growth picture significantly. Agar-gel diffusion plates with extracts of Novikoff cells and N-1 cells were set up against the resistant rat serum. No bands could be discerned from 10 such plates.

Irradiated Novikoff Cells as Antigen

There was a distinct possibility that the cells *in vitro*, after prolonged stay in an adverse environment, required a considerable readjustment of their metabolic process in order to proliferate when they were reinoculated into the hosts. Therefore, they might not be able to multiply at a rate that would overwhelm the host, thus allowing the host's defense mechanism to develop. However, not all tumors lose their virulence *in vitro* as did the Novikoff cells. Even with the Novikoff hepatoma, it took nearly a year before the cells *in vitro* failed to grow on reinoculation into susceptible hosts (*2*). If the requirement for successful protection was inoculation with *viable but nonproliferative tumor cells*, the use of irradiated malignant tumor cells taken directly from hosts might be expected to accomplish in a few hours the same protection as the use of tissue-culture cells.

The Novikoff tumor cells were collected and suspended in balanced salt solution. They were immediately irradiated with X rays at doses ranging from 800 to 6000 r. A part of each sample was cultivated *in vitro* to test whether the cells were viable after irradiation. At all dose levels, cells were observed to be living and to have membrane activity during the first 3 days.

Each test animal received 5 × 10^6 irradiated Novikoff tumor cells. It was noted that, beginning with 4000 r, there were no tumor takes. These animals were reinoculated with 5 × 10^6 Novikoff cells irradiated at

4000 r. Again no tumor developed. They were then challenged with nonirradiated Novikoff cells. Among 22 rats treated, only 5, or 23 percent, died of tumor.

During the period in which these experiments were being conducted, the articles of Donaldson and Mitchell (*5*) and McKee *et al.* (*6*) were published. Their data on immunizing mice against Ehrlich ascites tumor by inoculating irradiated tumor cells fully agree with ours.

DISCUSSION

There have been a number of reports of normal cells becoming transplantable after prolonged cultivation *in vitro* (*7–11*). In some instances the growth *in vivo* was indistinguishable from known neoplasms by any pathological criterion. These "normal" cell lines are heteroploid in their chromosomal constitution. In one report by Levan and Biesele (*12*) cytological change and transplantability were studied simultaneously. They observed a mouse embryonic cell strain that was classical diploid in the initial cultures gradually transformed to a double-stemline composition. However, the cells did not become transplantable as soon as the entire population had shifted its chromosomal pattern. After a number of transfers, fibrosarcoma was produced when the cells were introduced into mice of the strain of origin. This demonstrated that chromosomal changes alone do not necessarily cause the cells to become "neoplastic." This also agrees with the fact that occasional aneuploid cells can be found in normal tissues and yet not always suggest a process of carcinogenesis. It seems that only certain types of changes in the genetic background, and consequently in the metabolic properties, may lead to formation of cancer.

It is not within the scope of this study to speculate on the basic changes involved in carcinogenesis, but it is not entirely unreasonable to suggest that a tumor cell may lose its malignancy by further genetic alterations. The genetic make-up that is necessary for autonomous growth may be lost, suppressed, or altered when chromosomes have been reshuffled, duplicated, or deleted. The process is, perhaps, the Boveri concept (*13*) in reverse. Therefore, superficially the chromosome pattern of a derived tumor cell may have deviated more from the normal somatic complement than the original neoplasm; yet such a genome may actually be less malignant or nonmalignant.

The reduction of transplantability of the Novikoff hepatoma was not a new phenomenon. Several such examples are scattered through the literature. The L cells of the mouse, for instance, capable of producing sarcoma in over 90 percent of the C3H mice inoculated during the early period of the strain's history, reduced its transplantability to about 1 percent after further cultivation (*14*). Whether this change was also accompanied by chromosomal alterations cannot be traced, however. The lymphosarcoma MB3 of the mouse also failed to produce tumors

after prolonged stay in tissue culture (*15*). DeBruyn and Hansen-Melander (*16*) reported the cytological irregularities of this line. Dawe, Potter, and Leighton (*17*) found a progressive decrease of virulence of a reticulum-cell sarcoma grown in culture, which had changed the chromosomal constitution from near-diploid to hypotetraploid.

More interesting is the rat hepatoma studied by a group of Japanese investigators (*18*). After chromosomal transformation, the cells were still able to produce tumors when reinoculated, if they had been grown in stationary cultures. Conversely, when the cells had been grown in suspension cultures, they failed to produce tumors under otherwise identical conditions (*19*). The reasons for such a remarkable difference are not known.

Transplantation experiments involving human cell lines have been, of course, more difficult to perform. With volunteers from among cancer patients and prisoners, Southam, Moore, and their associates were able to register limited advances (*20*, *21*). Their findings were, in essence, very similar to ours with the Novikoff hepatoma. Immune mechanisms developed in normal males after inoculation of cells from such cancer lines as HEp2 and HEp3. These authors also showed some cross-immunity from one cell line to another, though it was not as strong as the immunity developed against the same cell strain.

Révész (*22*) attempted to immunize mice by inoculating them with X-irradiated Ehrlich tumor cells together with viable, nonirradiated cells. It is possible that various methods of reducing the virulence of a tumor can be applied, as long as they achieve the same purpose, *i.e.*, to obtain a population of nonvirulent but viable tumor cells. Recently, Goldin and Humphreys (*23*) succeeded in partially immunizing the mice against L1210 leukemia by treating the tumor cells with derivatives of amethopterin.

Transplantation experiments involving human and rat cells have one handicap, namely, the cells and hosts are not isogenic. In homotransplantation, development of immunity is so well known that it requires no citation. The same situation exists with the Ehrlich tumor (*5*, *6*) which is not strain-specific and has a long transplantation history. It would be interesting to know whether isogenic spontaneous tumors would behave similarly, since Hirsch *et al.* (*24*, *25*) suggested that there may be a possibility of immunizing an animal with its own tumor.

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Lung Tumors Produced by Intratracheal Inoculation of Polyoma Virus in Syrian Hamsters[1]

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SUMMARY

Proliferative lesions of bronchiolar and alveolar-lining cells, and tumors with histologic features of bronchiolar carcinoma, alveolar-cell carcinoma, and squamous-cell carcinoma have been produced in the lungs of hamsters inoculated intratracheally with polyoma virus. The areas of squamous-cell carcinoma appeared to arise in bronchioles and intrapulmonary bronchi; however, no evidence of origin from trachea or main bronchi was found. A transplantable squamous-cell carcinoma has been derived from one of the lung tumors and has retained squamous differentiation for four transplant generations. Attempts to isolate polyoma virus from one of the lung tumors and from the transplanted squamous-cell carcinoma have been unsuccessful. The possible significance of these findings in the search for etiologic factors in human lung cancer is discussed.—J. Nat. Cancer Inst. 25: 937–965, 1960.

INCREASING INTEREST in the problem of carcinoma of the lung in man has been accompanied by efforts to reproduce the disease in laboratory animals. Della Porta and his associates have recently summarized some of the methods used to produce lung tumors in mice, rats, and rabbits, and have presented their own work on the induction of tracheobronchial carcinomas in the Syrian hamster by intratracheal and intrabronchial instillation of 9,10-dimethyl-1,2-benzanthracene[4] (*1*). They stated that hamsters were suitable animals for studies of lung carcinogenesis, since spontaneous lung tumors were never observed in several hundred hamsters studied in their laboratory, and pulmonary infections were uncommon in untreated animals. Because it has been shown that subcutaneous inoculation of polyoma virus can produce tumors in Syrian hamsters (*2*), we decided to study the effect of intratracheal inoculation of polyoma virus in these animals.

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[4] *Chemical Abstracts'* nomenclature: 7,12-Dimethylbenz[*a*]anthracene.

MATERIALS AND METHODS

Animals.—Weanling male Syrian golden hamsters were obtained from the Animal Production Section of the National Institutes of Health. They were housed in plastic cages and divided into groups of 4 or 5 in each cage. They were fed Purina laboratory chow daily, greens and apples 3 times a week, and given water *ad libitum.*

Virus.—The strain of polyoma virus used in these studies was initially isolated in cultures of milk-adapted P388 D_1 cells from a cell-free filtrate of a parotid-gland tumor extract from a C3H/Bi mouse (*3*). For the present experiments, virus pools from the tenth and fourteenth serial passages in milk-adapted P388 D_1 cells were used. All virus preparations were stored in glass test tubes with rubber stoppers at −20° C. in an electric freezer. The pools were titrated by serial tenfold dilutions in milk-adapted P388 D_1 cells. The tenth passage pool contained $10^{4.5}$TCID50 per 0.25 ml. and the fourteenth passage pool contained $10^{6.5}$TCID50 per 0.25 ml.

Control fluids.—Sterile tissue-culture medium (20% autoclaved nonfat milk and 80% mixture #199), and medium and cells from cultures of P388 D_1 cells free of polyoma virus were used to inoculate control animals. All control fluids were frozen and thawed once before use.

Inoculation procedure.—Animals were anesthetized by intraperitoneal injection of chloral hydrate. A midline incision was made in the neck and the trachea was exposed. One ml. of virus preparation or control fluid was injected directly into the tracheal lumen with a syringe and a #20 needle. The incision was closed with metal clips.

Twenty-two animals were inoculated with virus preparations, 5 with sterile tissue-culture medium, and 9 with medium and cells from P388 D_1 cultures free of virus. Four of the 22 animals inoculated with virus preparations received undiluted fourteenth passage pool (12,800,000 TCID50); 5 received a 1:5 dilution of fourteenth passage pool (2,560,000 TCID50); 4 received a 10^{-2} dilution of fourteenth passage pool (128,000 TCID50); 5 received undiluted tenth passage pool (128,000 TCID50); and 4 received a 10^{-3} dilution of fourteenth passage pool (12,800 TCID50), (table 1). Virus dilutions were made in milk medium (20% autoclaved nonfat milk and 80% mixture #199).

Postmortem studies.—All internal organs of each animal that died or was killed were examined. Tissues for histologic sections were fixed in Zenker's solution with 10 percent formalin. The lungs and tracheobronchial tree were cut into multiple blocks and all were embedded for sectioning and histologic study. Representative sections of the following organs were also examined histologically: salivary glands, larynx, thyroid gland, parathyroid glands, thymus gland, heart, liver, gallbladder, spleen, pancreas, kidneys, adrenal glands, urinary bladder, testes, ovaries, uterus, lymph nodes, skeletal muscle, and sternal bone marrow. Sections were stained routinely with hematoxylin and eosin, and selected sections were stained with mucicarmine and periodic acid-Schiff with and without diastase digestion.

Transplantation of tumors.—Three lung tumors were selected for transplantation. The tumors were removed from the lungs with sterile instruments and placed in sterile petri dishes. The tissue was minced into 1 to 2 mm. fragments and 3 to 4 of these were placed in a #12 trocar. The recipients were weanling male hamsters approximately 28 days old. The tissue for transplantation was inserted subcutaneously in the right axillary region.

RESULTS

Of the 22 hamsters inoculated intratracheally with polyoma virus, 6 were still alive after 400 days of observation (table 1). Sixteen animals died and all were autopsied (table 2). On gross examination, tumors were found in the lungs of 6 of the 16. The tumors had histologic features of bronchiolar carcinoma, alveolar-cell carcinoma, and squamous-cell carcinoma. Of the 10 animals with no lung tumors evident on gross examination, proliferative lesions of bronchiolar and alveolar-lining cells were observed microscopically in 5.

TABLE 1.—Syrian hamsters inoculated intratracheally with polyoma virus

Group	Number of animals	Inoculum	Number living 400 days after inoculation	Number autopsied	Number with lung tumors or pulmonary epithelial proliferative lesions
A	4	12,800,000 TCID50* (14th passage)	0	4	4
B	5	2,560,000 TCID50 (14th passage)	0	5	3
C	4	128,000 TCID50 (14th passage)	2	2	1
D	5	128,000 TCID50 (10th passage)	1	4	3
E	4	12,800 TCID50 (14th passage)	3	1	0
Total	22		6	16	11

*Tissue-culture infectious doses.

The 5 control animals inoculated intratracheally with milk medium were killed 1 year after inoculation and no pulmonary lesions were found. One of the 9 control animals inoculated with milk medium and cells from P388 D_1 cultures was killed after 20 months and no pulmonary lesions were found. The other animals in this control group are still alive and will be observed until they die.

Lung Tumors

On gross examination, the lungs of 6 of the animals (#3, 4, 9, 11, 13, and 14) had multiple white nodules 2 to 8 mm. in diameter in all or nearly all lobes. Pink and tan cystic masses 1 to 2 cm. in diameter were also present in the lungs (fig. 1). The cut surfaces of the nodules were glistening and

TABLE 2.—Autopsy findings in Syrian hamsters inoculated intratracheally with polyoma virus

Animal number	Group	Age at inoculation (*days*)	Age at death (*days*)	Pulmonary lesions: Bronchial and alveolar cell proliferation	Pulmonary lesions: Lung tumors	Pulmonary lesions: Angiomatous lesions	Liver angiomas	Subcutaneous sarcomas at tracheotomy sites
1	A	38	137	+	−	−	−	−
2	A	38	233	+	−	−	+	+
3	A	38	330	−	+	−	−	−
4	A	38	429	−	+	−	−	−
5	B	26	53	+	−	+	+	−
6	B	26	67	+	−	+	+	−
7	B	26	74	−	−	−	+	−
8	B	26	116	−	−	+	+	−
9	B	26	190	−	+	−	+	−
10	C	29	86	−	−	−	+	−
11	C	29	338	−	+	−	−	−
12	D	30	176	+	−	−	+	−
13	D	30	267	−	+	−	−	−
14	D	30	331	−	+	−	−	−
15	D	30	349	−	−	−	+	+
16	E	26	336	−	−	−	−	−

white. The cystic masses were filled with turbid, red-brown fluid and lined by red and tan, granular tissue. The tracheobronchial lymph nodes were not enlarged and no gross evidence of tumor metastases in lymph nodes was found. One of the animals (#9) had a hemoperitoneum caused by bleeding from an angiomatous tumor of the liver. The other 5 animals with lung tumors had no gross evidence of tumors in other organs.

Microscopic examination of the lungs showed that the tumor nodules replaced considerable amounts of pulmonary parenchyma (fig. 2) and contained areas classified histologically as bronchiolar carcinoma, alveolar-cell carcinoma, squamous metaplasia of bronchiolar and bronchial epithelium, and squamous-cell carcinoma. Frequently, all these histologic types were seen within a single nodule.

In the areas classified as bronchiolar carcinoma, the bronchioles were dilated and partially or completely lined by columnar cells with large, pleomorphic vesicular and hyperchromatic nuclei and small to moderate amounts of pale, eosinophilic cytoplasm. Mucin was present in the cytoplasm of some of these cells (fig. 3). Alveolar ducts and alveoli adjacent to such bronchioles were lined by similar cells (fig. 4), and papillary projections of these cells into dilated alveolar ducts and alveoli were seen. Polymorphonuclear leukocytes and eosinophilic granular material frequently filled the bronchioles in the tumor nodules, and all the nodules had areas of necrosis with polymorphonuclear leukocytic infiltration.

Many of the alveoli in the tumor nodules were lined by and filled with cuboidal and low columnar cells with pleomorphic hyperchromatic nuclei (figs. 5 and 6) and with no mucin demonstrable in their cytoplasm or in

the alveolar spaces. Mitoses were not numerous and no intracytoplasmic or intranuclear inclusion bodies were seen. In our opinion, these tumor cells probably arose from alveolar-lining cells, and the areas have been classified histologically as alveolar-cell carcinoma. The possibility that these tumor cells arose from terminal bronchiolar epithelium, however, cannot be eliminated by study of the material available in the present experiments.

In many tumor nodules there were foci of well-differentiated, stratified squamous epithelium. These often lined dilated bronchioles and, less frequently, were in the mucosa of intrapulmonary bronchi. In some areas, the squamous cells had minimal nuclear pleomorphism and hyperchromatism, and there was no evidence of invasion of adjacent pulmonary parenchyma. Such foci were classified as squamous metaplasia rather than as squamous-cell carcinoma. In other areas, the squamous epithelium had mild to moderate degrees of cellular pleomorphism, but there was no definite evidence of invasion of surrounding tissues (fig. 7). These areas were classified as squamous metaplasia with cellular atypism; however, some had histologic features which might also be consistent with a diagnosis of noninfiltrating, well-differentiated squamous-cell carcinoma.

In addition to the foci classified as squamous metaplasia, however, there were areas with histologic features of infiltrating squamous-cell carcinoma (fig. 8). These often appeared to originate in bronchioles and, less frequently, in intrapulmonary bronchi; bronchioles and intrapulmonary bronchi sometimes could be traced into large areas of squamous-cell carcinoma (fig. 9). The centers of areas of squamous-cell carcinoma often had collections of keratin, polymorphonuclear leukocytes, and eosinophilic and basophilic granular material. Nests of squamous tumor cells were frequently seen adjacent to areas of alveolar-cell carcinoma in the tumor nodules (fig. 10).

The cystic masses in the lungs were filled with keratin, polymorphonuclear leukocytes, and granular debris, and lined by stratified squamous epithelium which was often well differentiated with little cellular atypism and no evidence of infiltration of underlying lung (fig. 11). In some areas, however, the squamous epithelium lining the cysts had histologic features of well-differentiated noninfiltrating squamous carcinoma (fig. 12). In other areas, the lining epithelium had histologic features of poorly differentiated squamous carcinoma (fig. 13) and nests of squamous tumor cells invaded the adjacent lung. Smooth muscle was present in the walls of some cysts, and dilated bronchioles and intrapulmonary bronchi partially lined by stratified squamous epithelium occasionally could be traced into the cysts. We therefore think that the cysts may be the result of marked dilatation of bronchioles and intrapulmonary bronchi lined by metaplastic and neoplastic stratified squamous epithelium.

In several animals, the stroma associated with areas of carcinoma had histologic features of fibrosarcoma (fig. 14). These areas resembled the sarcomatous lesions produced in the subcutaneous tissues of hamsters by subcutaneous inoculation of polyoma virus, and they also were similar

to the intracranial sarcomas produced by intracerebral inoculation of hamsters with polyoma virus (*4*).

In one autopsied animal (#3), thick, red-yellow fluid was found in both pleural cavities. Granular white material covered the parietal and visceral pleural surfaces, and all lobes of the lungs contained multiple tumor nodules. Microscopically, collections of pleomorphic cells with hyperchromatic and vesicular nuclei and moderate amounts of eosinophilic cytoplasm were seen on the pleural surfaces (fig. 15). Multinucleated giant cells were numerous, and bizarre distorted nuclear forms were common. Transition from mesothelial cells of the pleura to tumor cells was seen, and it was our opinion that the lesion was a mesothelioma of the pleura. The possibility, however, that it represented extensive pleural metastases from a poorly differentiated area of primary lung tumor could not be eliminated. In some areas, pleomorphic tumor cells thought to be of mesothelial origin were present on the pleural surface, while well-differentiated squamous-cell carcinoma, apparently arising in bronchioles, infiltrated the underlying lung (fig. 16).

There was no evidence of primary origin of squamous-cell carcinoma from the trachea or main bronchi. There were, however, foci of squamous metaplasia with epithelial atypism in the mucosa of main bronchi in one animal. Although a search for metastases from the lung carcinomas was made microscopically, none were found in tracheobronchial lymph nodes or at any other sites. The angiomatous tumor in the liver of hamster #9 was similar microscopically to the vascular liver tumors previously described by Eddy and her associates in hamsters inoculated subcutaneously with polyoma virus (*2*).

Transplantation of Lung Tumors

Three attempts were made to transplant lung tumors subcutaneously to weanling hamsters. The lung tumor from hamster #13 did not grow on transplantation. Tumor tissue was transplanted to 3 weanling hamsters and there were no palpable masses at the transplant sites during a 6-month period of observation.

A second attempt was made with the lung tumor from animal #14. Nodules were palpable at the transplant sites in 2 weeks, and after 4 weeks they measured 2 cm. in diameter. On gross examination, they were composed of soft, pink tissue with a few small, cystic areas. Microscopically, they were predominantly sarcomatous and resembled the sarcomatous areas in some lung tumors. Scattered in the sarcomatous tissue of the transplant were occasional duct-like structures lined by columnar and cuboidal epithelial cells with pleomorphic hyperchromatic nuclei, resembling the bronchioles lined by neoplastic cells in the primary lung tumors (fig. 17). The cystic areas in the transplant were lined by tissue with histologic characteristics of squamous-cell carcinoma. The tumor maintained this mixed histologic appearance in two consecutive transplant generations, with predominance of the sarcomatous elements. Transplantation of this tumor line was then discontinued.

The third attempt at transplantation was made with the lung tumor from animal #11. Fragments of tumor tissue from the lining of cystic masses were selected, and 5 days after transplantation, small tumors were palpable at the transplant sites. These grew rapidly, and after 20 days measured 2 cm. in diameter. One animal was killed and the tumor was transplanted to 3 other animals. Grossly, the transplanted tumor was a cystic structure filled with turbid, red-brown material and lined by granular, tan tissue. Microscopically, the cyst wall was lined by tissue with histologic features of squamous-cell carcinoma. The tumor cells had large, pleomorphic hyperchromatic nuclei and mitoses were numerous (fig. 18). Keratinization was present though not prominent, and intercellular bridges could be identified. This squamous-cell carcinoma has now been transplanted for 4 generations. It continues to form cysts and has histologic features of epidermoid differentiation.

Attempts to Isolate Virus From Tumors

One attempt was made to isolate polyoma virus from a primary lung tumor. The tumor from animal #13 was removed with aseptic technique and frozen at −20° C. It was subsequently thawed and a 20 percent suspension of tumor tissue in milk medium was prepared. This material was inoculated into tube cultures of milk-adapted P388 D_1 cells. Three tubes were inoculated with undiluted 20 percent tumor suspension and two tubes were inoculated with a 10^{-1} dilution of this material. The tubes were observed for 40 days with no evidence of cytopathic effect. With similar methods, attempts to recover virus from the transplanted tumors have thus far been unsuccessful.

Bronchiolar and Alveolar Cell Proliferations

Five of the 10 animals with no lung tumors evident on gross examination had microscopic lesions classified as bronchiolar and alveolar-lining cell proliferations. Some of these lesions consisted only of collections of atypical epithelial cells in the bronchiolar mucosa projecting into the bronchiolar lumens (fig. 19). In others, alveolar ducts and alveoli adjacent to the bronchioles were lined by and filled with cuboidal and columnar cells with pleomorphic vesicular and hyperchromatic nuclei. The bronchiolar and alveolar cell proliferations resembled areas of bronchiolar and alveolar-cell carcinoma in the lung tumors, but they were smaller, measuring less than 1 mm. in diameter. Since the lesions were not recognized grossly, none were transplanted.

Hamsters #5, 6, and 12 with these lesions died with hemoperitoneum caused by bleeding from angiomatous tumors of the liver, a phenomenon frequently observed in hamsters inoculated subcutaneously with polyoma virus. Animals #5 and 6 had multiple angiomatous lesions of the lungs, similar to those described by Eddy and her associates in hamsters inoculated subcutaneously with the virus (*2*). These animals also had multiple liver-cell emboli from the angiomatous hepatic tumors in capillaries of

the lungs and within the angiomatous pulmonary lesions (fig. 20). Kupffer cells with large hyperchromatic nuclei sometimes could be identified in these liver-cell emboli.

DISCUSSION

In recent years most of the studies of the etiology of lung cancer in laboratory animals and in man have been concerned with carcinogenic hydrocarbons, tars, and metals. There has been some interest in the possible viral etiology of pulmonary adenomatosis, and lesions resembling human pulmonary adenomatosis have been produced by inoculation of squirrel fibroma virus into newborn squirrels (*5*). The neoplastic nature of such lesions, however, has not been established and the relationship of pulmonary adenomatosis to terminal bronchiolar and alveolar-cell carcinomas of the lung is not clear.

The discovery of the mouse polyoma virus, some of its biological and biochemical properties, and the histopathology of the tumors produced by it have been reviewed by several authors (*6–9*). Eddy and associates (*2*) have described undifferentiated lung tumors arising in hamsters inoculated subcutaneously with the polyoma virus on the day of birth. Stewart, Eddy, and Stanton (*6*) have described nuclear enlargement and margination of chromatin in bronchial-epithelial cells and alveolar-lining cells of mice inoculated subcutaneously with polyoma virus. Dawe and associates (*8*) observed proliferative lesions of the terminal bronchiolar epithelium in mice inoculated subcutaneously on the day of birth. In the studies reported here, lung tumors with histologic features of bronchiolar carcinoma, alveolar-cell carcinoma, and squamous-cell carcinoma have been produced by intratracheal inoculation of polyoma virus in weanling hamsters.

As yet, the role of the polyoma virus in the production of these tumors is not clear. The failure to demonstrate virus in the one lung tumor tested and in the transplanted squamous-cell carcinoma is in accord with reports of other investigators on the difficulties encountered in recovering virus from sarcomatous hamster tumors produced by polyoma virus (*10, 11*). Vogt and Dulbecco have proposed that the virus may transform a normal cell into a tumor cell and become integrated into the tumor cell in a manner like that found in lysogenic bacterial cultures (*12*). The polyoma virus may thus exist in the tumor cells as a provirus. If this is true, it may be possible to recover virus from the lung tumors by growing them in tissue culture and exposing the cultures to ultraviolet or ionizing radiations.

An alternate hypothesis to that of an integrated virus in the tumor cell is that the virus causes a neoplastic transformation of bronchial, bronchiolar, and alveolar-lining cells and is thereafter unnecessary for continued tumor growth. It therefore may be impossible to recover virus from the tumors by any method, despite the fact that they were produced by the virus.

A third possibility is that the tumors are not produced by the virus but rather by some chemical substance liberated into the culture fluids during viral synthesis. The accumulation of an abnormal ribonucleoprotein in P388 D_1 cells infected with polyoma virus has been demonstrated cytochemically (*13*). Since evidence exists that polyoma virus is a deoxyribonucleic acid (DNA) virus (*9*), it is unlikely that the abnormal ribonucleoprotein is a constituent of the viral particle. It is possible, however, that such an abnormal nucleoprotein or some other nonviral substance produced during viral synthesis might act as a chemical carcinogen when instilled into the tracheas of hamsters. The controls in the present study do not eliminate this possibility, and additional experiments designed to test this hypothesis are planned.

The histopathogenesis of the lung tumors and their relationship to the lesions classified as bronchiolar and alveolar-lining-cell proliferations cannot be precisely determined from the present material. Adequate study of these problems would require an experiment in which animals were inoculated with virus intratracheally and subsequently killed at appropriate intervals for pathologic examination (*14*). It seems probable, however, that the microscopic lesions classified as bronchiolar and alveolar-cell proliferations can progress to tumors with histologic features of bronchiolar carcinoma and alveolar-cell carcinoma.

The origin of squamous-cell carcinoma in the tumor nodules and cysts was not always evident histologically; however, it often appeared to arise in dilated bronchioles and intrapulmonary bronchi. Since squamous metaplasia was seen in similar sites, it is possible that there was progression from squamous metaplasia to squamous-cell carcinoma in the mucosa of bronchioles and intrapulmonary bronchi. There was no evidence of origin of squamous-cell carcinoma from the trachea or main bronchi; however, this may be a result of inadequate exposure of the mucosa of these structures to the viral inoculum.

The significance of the present work in relation to studies of etiologic factors in human lung cancer is difficult to assess at this time. If some human lung cancers are caused by viruses, our experimental work suggests that it may be difficult or impossible to recover virus from those tumors. If suitable methods can be devised for recovering polyoma virus from the lung tumors of hamsters or from the transplantable squamous-cell carcinoma derived from one of these tumors, such methods may also be useful for the demonstration of oncogenic viruses in human lung carcinomas. It is possible that the application of the large amount of information available on integrated virus-host cell relationships in lysogenic bacterial cultures may result in a solution to these challenging problems.

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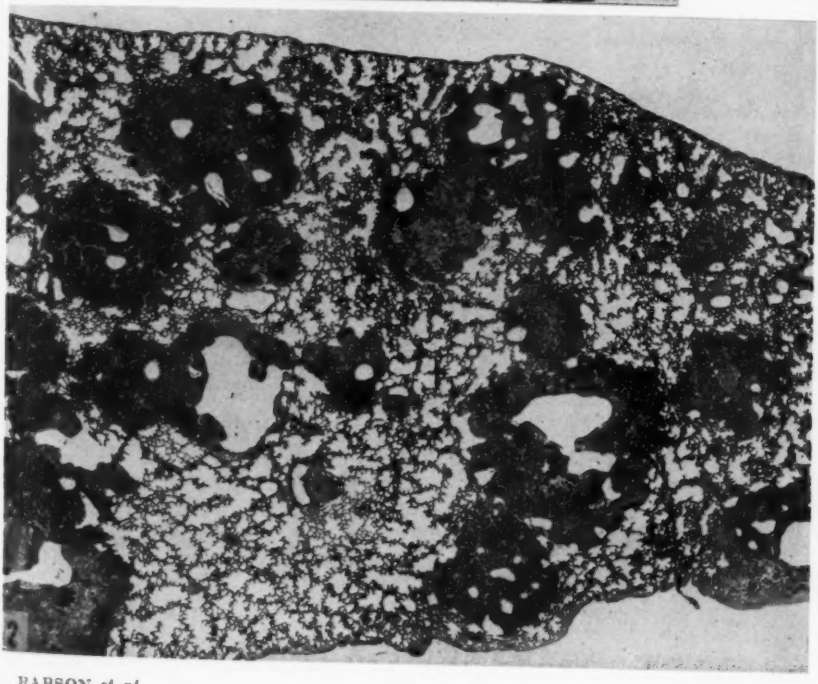
PLATE 126

FIGURE 1.—Large cystic tumor involving *lower* lobe of *left* lung (*arrow*) 301 days after intratracheal inoculation of polyoma virus. Multiple white nodules, 2 to 8 mm., were present in other lobes of lungs. Hamster #14.

FIGURE 2.—Multiple tumor nodules in lung of hamster 164 days after intratracheal inoculation of polyoma virus. Bronchioles in nodules are dilated, and adjacent alveoli are lined by and filled with tumor cells. Hamster #9. Hematoxylin and eosin. × 19



563711—60——5

PLATE 127

FIGURE 3.—Bronchiole from tumor nodule in lung of hamster, 164 days after intratracheal inoculation of polyoma virus. Partial replacement of bronchiolar epithelium by atypical cells. Mucin (*black*) is seen in the cytoplasm of many atypical cells. Hamster #9. Periodic acid-Schiff reaction after diastase digestion. × 430

FIGURE 4.—Area of tumor classified as bronchiolar carcinoma in lung of hamster, 164 days after intratracheal inoculation of polyoma virus. Alveoli are lined by pleomorphic columnar cells with mucin (*black*) in alveolar lumens. Hamster #9. Periodic acid-Schiff reaction after diastase digestion. × 780

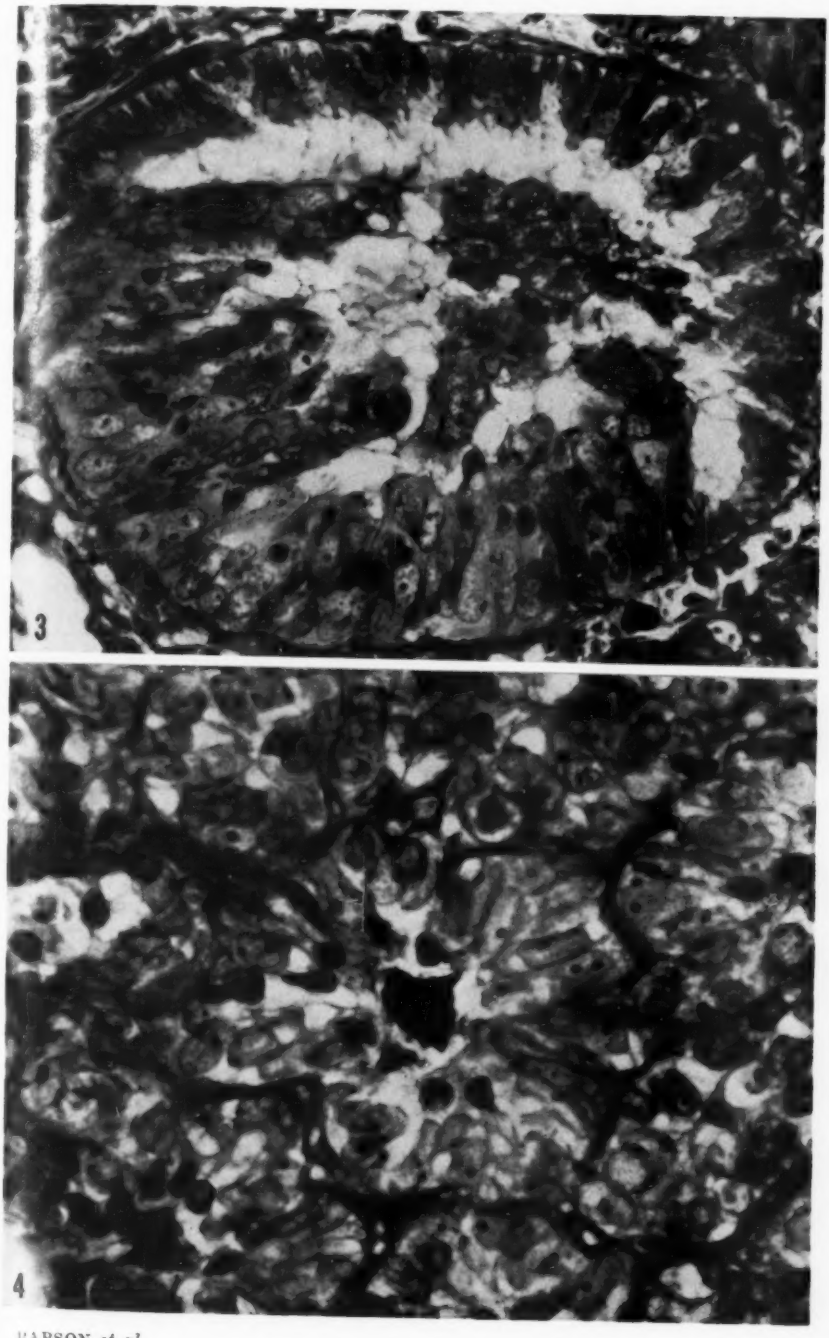

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PLATE 128

FIGURE 5.—Area of tumor classified as alveolar-cell carcinoma in lung of hamster, 309 days after intratracheal inoculation of polyoma virus. Alveoli are lined by large cuboidal cells with pleomorphic vesicular and hyperchromatic nuclei. Hamster #11. Hematoxylin and eosin. × 380

FIGURE 6.—Area of tumor classified as alveolar-cell carcinoma in lung of hamster, 309 days after intratracheal inoculation of polyoma virus. Alveoli are lined by and filled with cuboidal and columnar tumor cells. Hamster #11. Hematoxylin and eosin. × 380

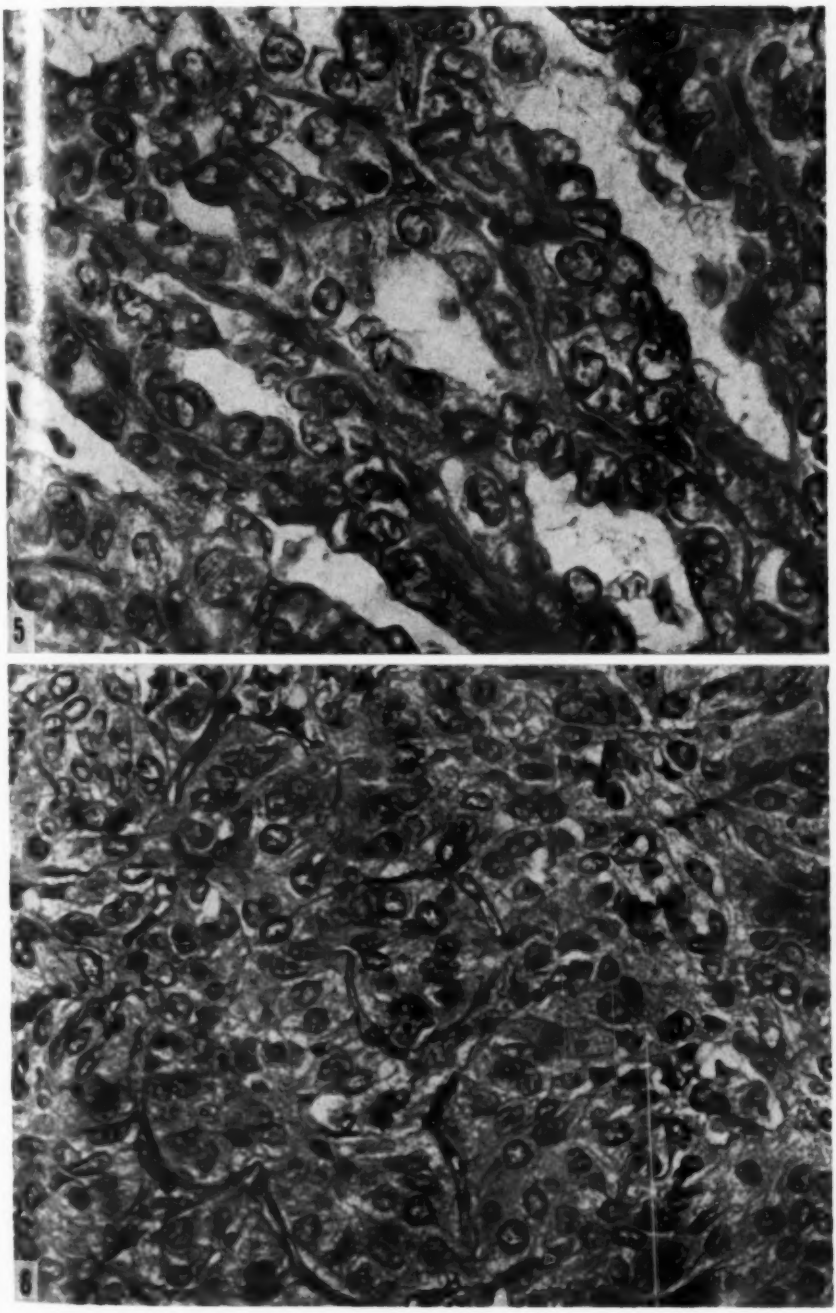
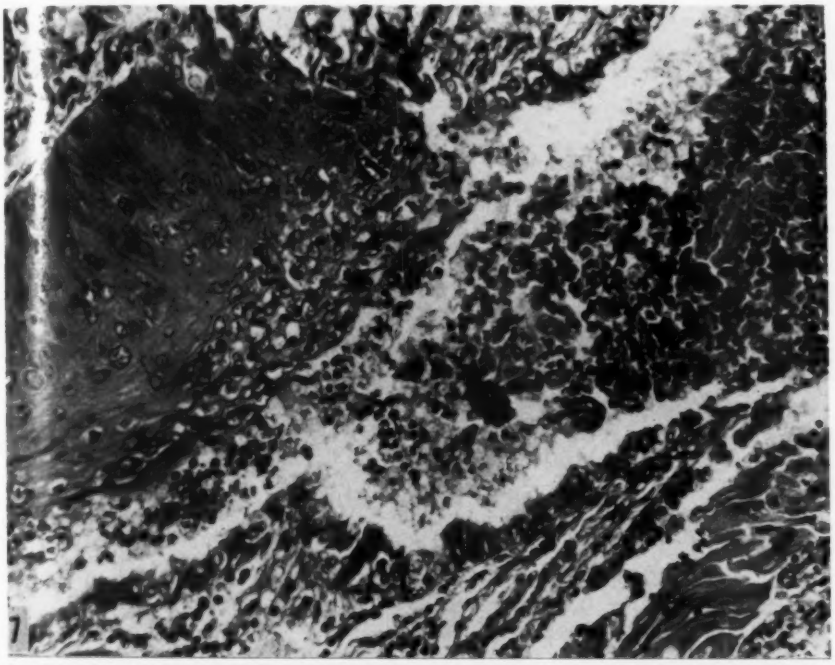


PLATE 129

FIGURE 7.—Dilated bronchiole partially lined by stratified squamous epithelium in lung of hamster, 301 days after intratracheal inoculation of polyoma virus. Cellular pleomorphism and nuclear enlargment in squamous epithelium. These areas were classified as squamous metaplasia with epithelial atypism. Hamster #14. Hematoxylin and eosin. × 260

FIGURE 8.—Area with histologic features of squamous-cell carcinoma in tumor nodule from lung of hamster, 164 days after intratracheal inoculation of polyoma virus. Cells have large hyperchromatic and vesicular nuclei and moderate amounts of eosinophilic cytoplasm. Dyskeratotic cells are present and intercellular bridges could be identified. Hamster #9. Hematoxylin and eosin. × 570



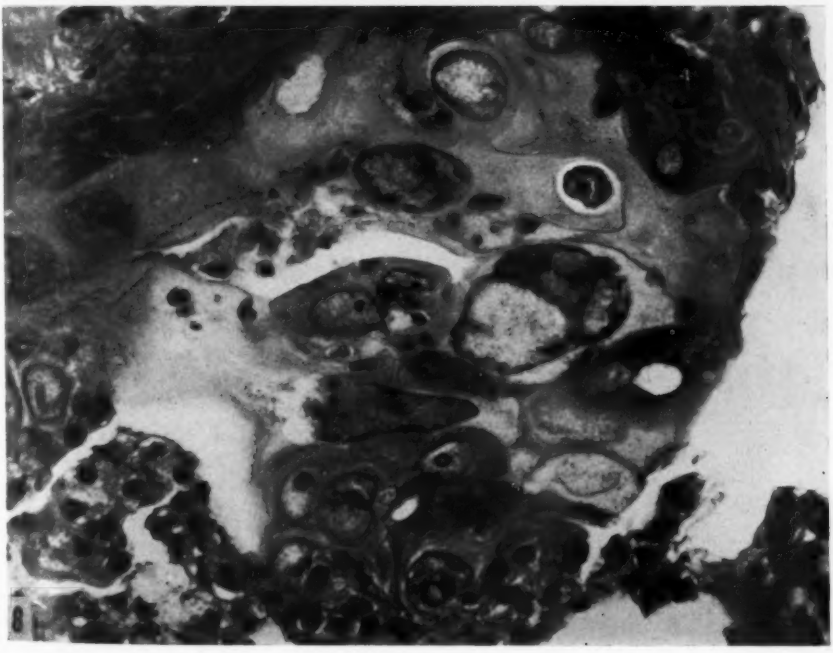
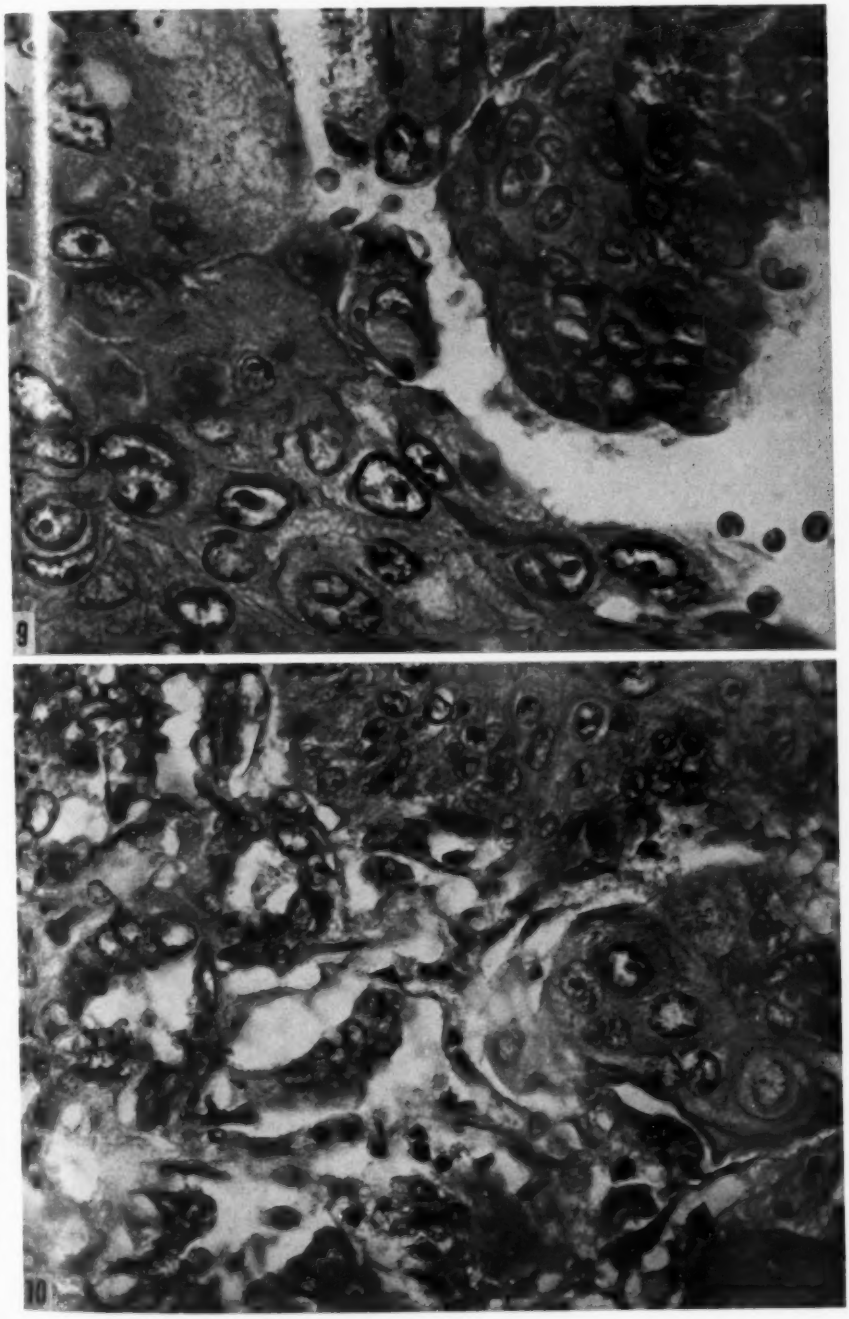


PLATE 130

FIGURE 9.—Bronchiole entering area of squamous-cell carcinoma in lung tumor of hamster, 309 days after intratracheal inoculation of polyoma virus. Bronchiolar epithelium is present in *upper right*. Hamster #11. Hematoxylin and eosin. × 380

FIGURE 10.—Tumor in lung of hamster, 301 days after intratracheal inoculation of polyoma virus. Area of squamous-cell carcinoma on *right side* is in contact with alveolar-cell carcinoma on *left*. Hamster #14. Hematoxylin and eosin. × 870



563711—60——6

PLATE 131

FIGURE 11.—Wall of cystic tumor in lung of hamster, 301 days after intratracheal inoculation of polyoma virus. Cyst lumen in *lower portion* of photograph contains polymorphonuclear leukocytes, eosinophilic and basophilic granular debris, and keratin. Cyst is lined by well-differentiated stratified squamous epithelium with mild to moderate cellular atypism but no evidence of infiltration of underlying stroma. Tumor nodules with histologic features of alveolar cell and bronchiolar carcinoma are in lung adjacent to cyst wall. Hamster #14. Hematoxylin and eosin. × 25

FIGURE 12.—Lining epithelium of cystic tumor in lung of hamster, 301 days after intratracheal inoculation of polyoma virus. Cells of the stratified squamous epithelium have large pleomorphic hyperchromatic nuclei. There is no evidence of infiltration of underlying tissue by squamous nests. This type of epithelium was classified as noninfiltrating, well-differentiated squamous-cell carcinoma. Hamster #14. Hematoxylin and eosin. × 520

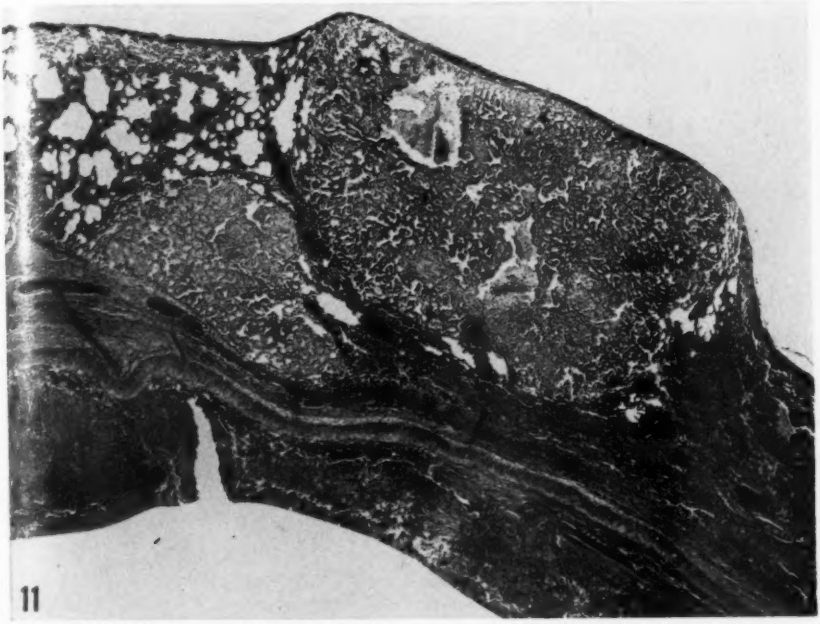

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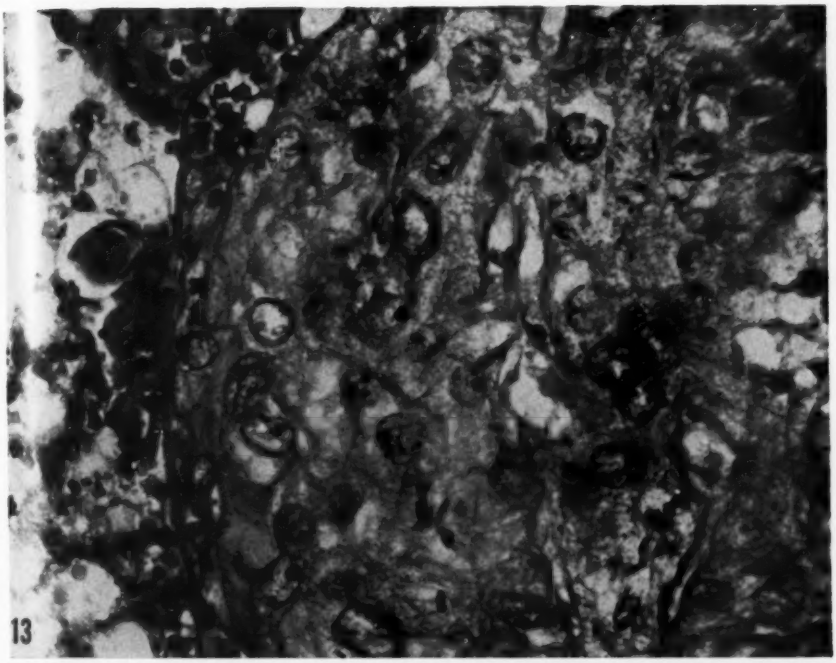
PLATE 132

FIGURE 13.—Area of squamous-cell carcinoma lining cystic tumor in lung of hamster, 301 days after intratracheal inoculation of polyoma virus. Epithelial cells have large pleomorphic, vesicular nuclei and moderate amounts of pale cytoplasm. Intercellular bridges were identified with difficulty and there was little evidence of keratinization. Infiltration of adjacent lung by nests of similar tumor cells was seen. Hamster #14. Hematoxylin and eosin. × 520

FIGURE 14.—Sarcomatous area in lung tumor of hamster, 309 days after intratracheal inoculation of polyoma virus. Portion of squamous-cell tumor is seen in the *upper right corner*. Hamster #11. Hematoxylin and eosin. × 260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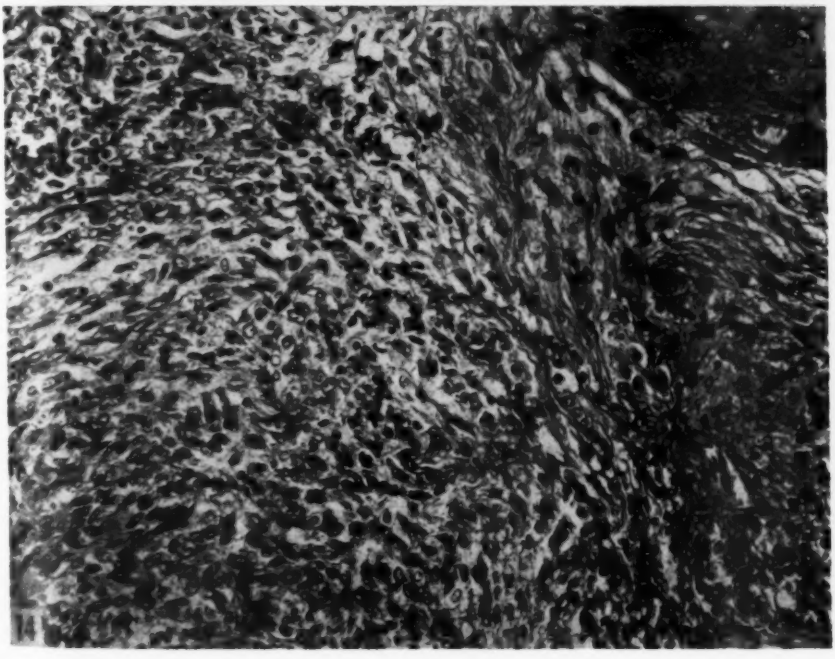
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PLATE 133

FIGURE 15.—Undifferentiated tumor, thought to be of mesothelial origin, on pleural surface of lung of hamster, 292 days after intratracheal inoculation of polyoma virus. Transition from mesothelial cells to these tumor cells was seen in some areas. Hamster #3. Hematoxylin and eosin. × 290

FIGURE 16.—Undifferentiated tumor, thought to be of mesothelial origin, on pleural surface with well-differentiated squamous-cell carcinoma in underlying pulmonary parenchyma in lung of hamster, 292 days after intratracheal inoculation of polyoma virus. Hamster #3. Hematoxylin and eosin. × 260

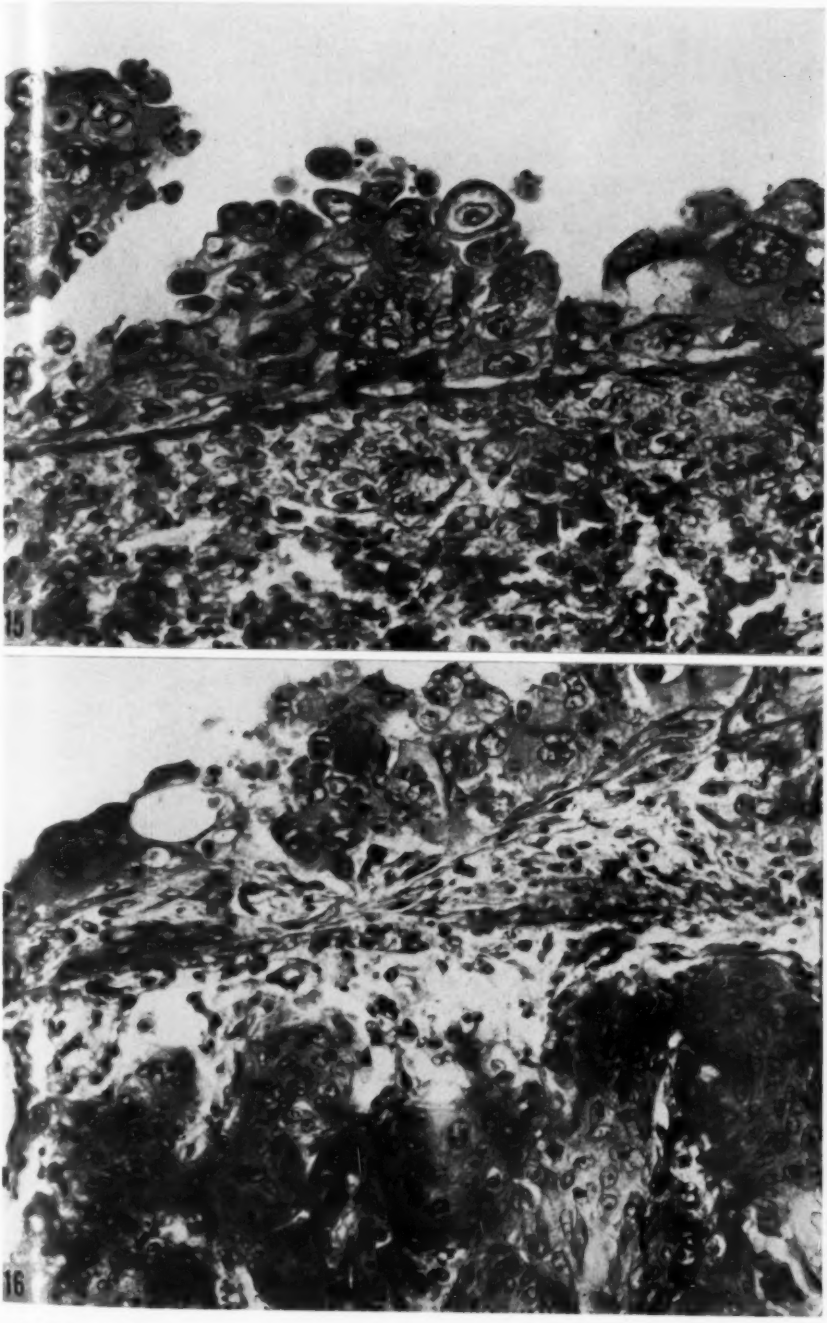
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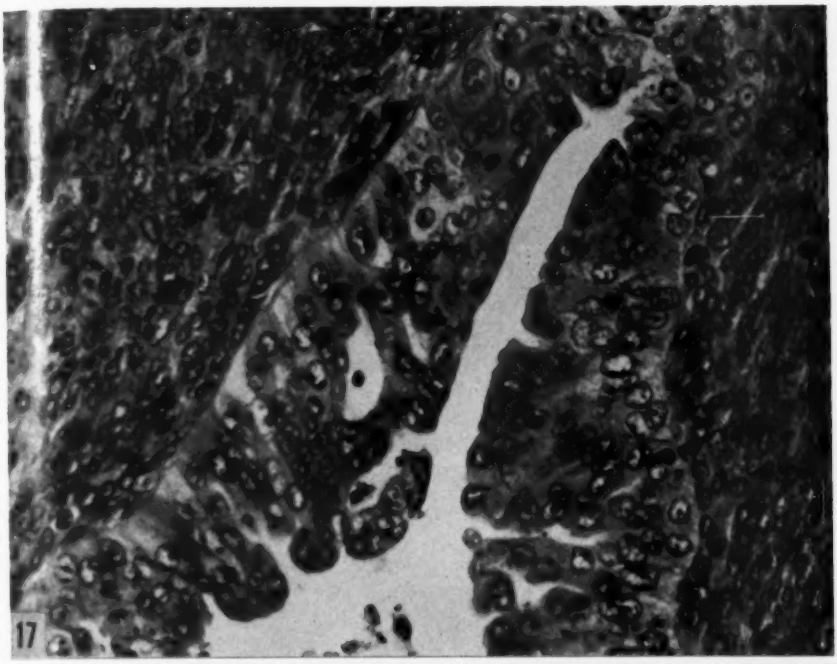
PLATE 134

FIGURE 17.—Second subcutaneous transplant generation of lung tumor from hamster #14. Transplanted tumor was composed predominantly of sarcomatous stroma; however, as shown in the photograph, there were a few epithelial structures which resembled the bronchioles lined by tumor cells seen in the original lung tumor. Hematoxylin and eosin. × 400

FIGURE 18.—Second subcutaneous transplant generation of squamous-cell carcinoma derived from lung tumor of hamster #11. Transplanted tumors were cystic and, microscopically, the lining was composed of pleomorphic cells with large hyperchromatic and vesicular nuclei and moderate amounts of pale, basophilic cytoplasm. Intercellular bridges and areas with keratinization were observed. Hematoxylin and eosin. × 380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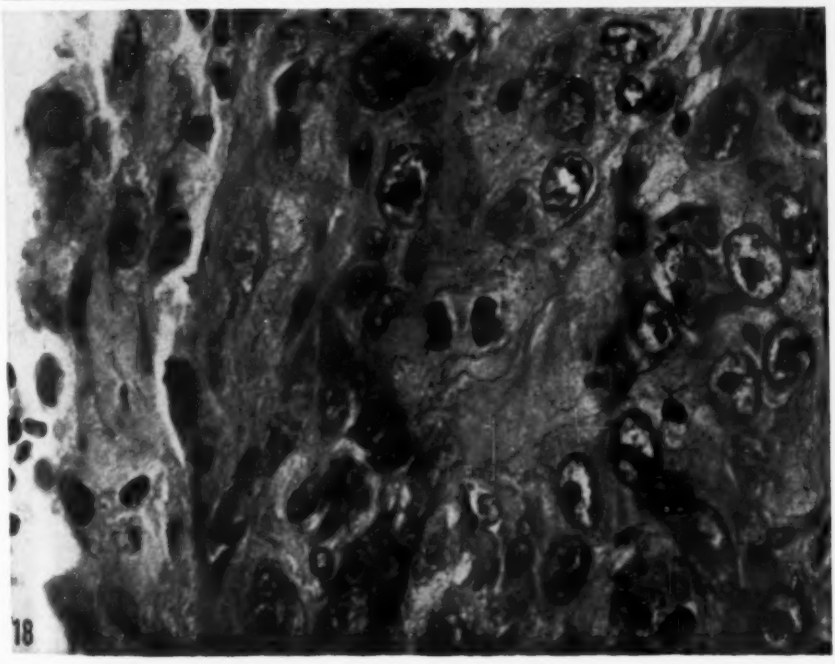
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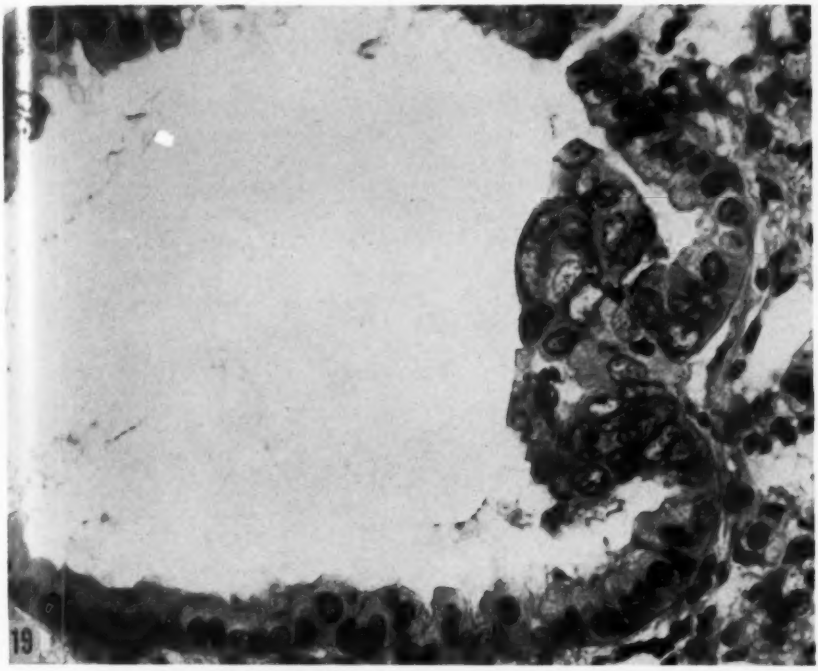
PLATE 135

FIGURE 19.—Proliferative lesion of bronchiolar epithelium in hamster, 27 days after intratracheal inoculation of polyoma virus. Atypical epithelial cells project into the bronchiolar lumen. These cells have pleomorphic hyperchromatic and vesicular nuclei. Hamster #5. Hematoxylin and eosin. × 610

FIGURE 20.—Angiomatous lesion in alveolar septum, 27 days after intratracheal inoculation of polyoma virus. Dilated, irregularly shaped vascular space is filled with erythrocytes and lined by endothelial cells with large nuclei and margination of chromatin along nuclear membranes. Cluster of liver cells presumably embolic from an angiomatous lesion of the liver is indicated by *arrow*. Hamster #5. Hematoxylin and eosin. × 430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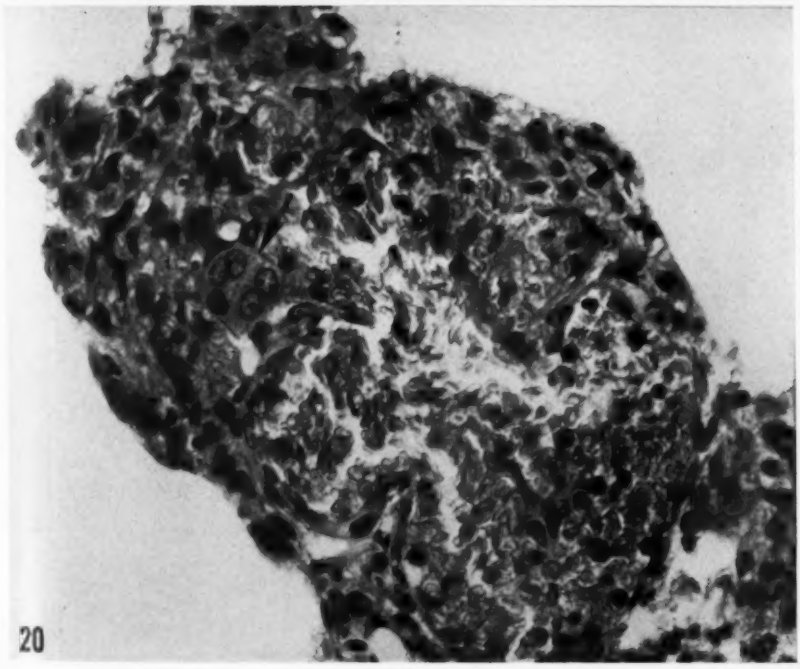
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Acid-Extractable Nuclear Proteins of Cancer Cells. I. Staining With Ammoniacal Silver [1, 2]

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SUMMARY

Studies of lymph-node responses disclosed that antigenic stimulation was associated with a change in ammoniacal-silver (A-S) staining of paraffin sections of lymph nodes. Sections of control lymph nodes lacked A-S staining of cell nuclei, but sections of lymph nodes responding to antigenic stimulation had A-S staining of the nuclei. Parallel changes were also seen in the nuclei of parenchymal cells. However, the nuclei of malignant lymphoid and parenchymal cells showed minimal or no A-S staining, regardless of the staining of the control homologous cells. These observations prompted a more detailed study of A-S staining of control and cancer cells. Smears of fresh tissues from human beings and mice were used. Under the conditions of study, A-S stained cell nuclei precisely. The chromatin structures of noncancerous cells were distinctly stained by A-S. In contrast, cancer cells had fine, silver-staining granules scattered with minimal structural orientation throughout the nuclei. Differential extraction studies indicated that A-S stained acid-extractable nuclear protein (AENP) whose physical characteristics and staining with alkaline Fast Green corresponded to that of a histone. Apparently, cancer cells are characterized by well-defined alterations in their AENP. However, further studies are needed to define the detailed physicochemical nature and biological significance of the observed changes in AENP.—J. Nat. Cancer Inst. 25: 967–989, 1960.

WE PREVIOUSLY reported that antigenic stimulation of mice and human beings was associated with a remarkable change in the ammoniacal-silver (A-S) staining of paraffin sections of the lymph nodes (*1–4*). Whereas the nuclei of control lymphoid cells were unstained by A-S, antigenic stimulation was followed by distinct staining of the nuclei of these cells. However, the nuclei of malignant lymphoid cells from patients with lymphosarcoma or Hodgkin's disease did not stain even when adjacent nonneoplastic lymphoid cells were stained (*5*). These observations, coupled with the fact that the lymphoid cells of the axillary lymph nodes of human fetuses (500–1500 gm.) were regularly stained with A-S, sug-

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gested that variations in A-S staining showed basic biological alterations in cells (*6*). These considerations justified a more detailed study of the A-S staining of control and cancerous tissues with particular reference to the biochemical basis of A-S staining.

This report describes differences between the A-S staining of parenchymal and cancer cells. Data will be presented which suggest that A-S stains an acid-extractable nuclear protein (AENP) having physicochemical and staining properties similar to a histone.

MATERIALS AND METHODS

Studies have been made on the A-S staining of parenchymal and carcinoma tissues from mice and human beings. Routinely prepared formalin-fixed tissue sections and smears of fresh tissues were used.

Routine tissue sections were obtained from the surgical and autopsy files of the Flower and Fifth Avenue Hospitals, New York. Sections were prepared from the tissues of CFW mice, with and without spontaneous mammary carcinomas and with and without prior injections of foreign proteins. The tissues were fixed in 10 percent sodium acetate-neutralized formalin, dehydrated in alcohol, cleared in chloroform, and embedded in paraffin. Sections were cut at 5 mμ, the paraffin was removed with xylol, and the slides were run through alcohol to water and stained in A-S. The A-S staining solution was prepared by adding a 10 percent aqueous solution of silver nitrate to concentrated ammonium hydroxide until a faint permanent turbidity was obtained. The slides were individually immersed with agitation in the A-S solution for approximately 5 seconds, washed with distilled water, and placed in a 3 percent solution of neutral formalin for 2 minutes. They were then washed in water, dehydrated in alcohol, cleared in xylol, and mounted in Permount.

Smears were prepared from a variety of fresh parenchymal and cancerous tissues obtained from human beings and mice. The smears were air-dried and then subjected to various types of fixation and extraction before staining with A-S.

RESULTS

Tissue Sections

Adult controls.—Liver and kidney sections were prepared from 20 healthy-appearing adult male and female CFW mice weighing more than 20 gm. These sections had no distinct staining of the nuclei of the parenchymal or stromal cells. In some there was a faint staining of the cytoplasm of the liver and kidney tubular cells (figs. 1 and 2).

Adult control mice injected with foreign protein.—Ten healthy-appearing male and female CFW mice weighing more than 20 gm. received sub-

cutaneous or intraperitoneal inoculations of 0.1 ml. of heparinized human blood, human serum, and diphtheria toxoid. Groups of 2 to 3 mice were killed 8, 24, 48, and 72 hours later. The liver and kidney sections from 8 of these mice had distinct staining of the nuclei by A-S (figs. 3 and 4).

Tumor-bearing control mice.—The liver, kidney, and tumor sections from 20 female CFW mice bearing spontaneous mammary carcinomas failed to stain with A-S.

Tumor-bearing mice, inoculated with foreign protein.—Ten female CFW mice bearing spontaneous mammary carcinomas approximately 1 cm. in diameter received injections of 0.1 ml. of heparinized human blood, human serum, and diphtheria toxoid. The mice were killed from 8 to 72 hours after the injection of antigen. The liver and kidney sections from 6 of these mice were characterized by A-S staining of the nuclei of the parenchymal and stromal cells. In contrast, all the nuclei of intermitotic cancer cells were either unstained or stained in the form of scattered fine granules. In dividing cancer cells, the chromosomes were frequently stained. However, the staining of the cancer-cell chromosomes was usually less intense than that of noncancerous cell chromosomes, *i.e.*, proliferating cells in secondary centers of reactive lymph nodes.

Surgical specimens.—Sections were obtained from cancerous and noncancerous tissues, removed in operations for cancer or for biopsy, namely, the breast, colon, liver, kidney, lung, lymph nodes, and bone marrow. Frequently the nuclei of the noncancerous tissue sections were not stained with A-S, but some staining of the nuclei and cytoplasm was noted. These stained tissues were taken predominantly from patients with a variety of systemic diseases or with such degenerative diseases as cirrhosis and glomerulonephritis (fig. 5). In some instances the nuclei of hyperplastic and adenomatous cells were stained with A-S.

Particularly notable was the absence of stain in the nuclear chromatin of the cancer cells, even when adjacent parenchymal cell nuclei were stained. In none of the cancerous tissues examined were the nuclei stained distinctly with A-S (fig. 6).

Autopsy.—To date we have examined liver and kidney sections obtained from more than 100 autopsies on human beings. The influence of various nonneoplastic diseases on the A-S staining of the nuclei of parenchymal cells will be reported in detail in a subsequent paper. A-S frequently did not stain the parenchymal cells of patients who died with noninfectious diseases such as myocardial infarct or cerebrovascular accident, but the nuclei of liver and kidney sections were commonly stained in a variety of disease conditions: cirrhosis, lupus erythematosus dissemenatus, and systemic infections (fig. 7). However, the intermitotic nuclei of cancer cells of diverse types, primary and metastatic, had minimal or no staining with A-S. It is pertinent to record our observations on a human hepatoma arising in a liver with postnecrotic cirrhosis and in hepatomas induced in rats by *N,N*-dimethyl-*p*-phenylazoaniline.[4] Most of the noncancerous

[4] The rat tissues were made available to us by Dr. Fred Zak, Mount Sinai Hospital, N.Y.

cells of the human and rat livers—hyperplastic-hepatic and bile-duct cells, Kupffer cells, and lymphocytes—exhibited distinct A-S staining of the nuclei. In contrast, the nuclei of the nondividing cancerous cells stained faintly or not at all. The chromosomes of dividing cancer cells were usually stained with minimal to moderate intensity.

The data obtained in the studies of sections of parenchymal and cancerous tissues from human beings and mice may be summarized as follows: The nuclei of parenchymal cells of human beings and mice were not usually stained with A-S. However, antigenic stimulation or systemic diseases of certain types might be associated with distinct silver staining of the nuclei of liver and kidney and other parenchymal and stromal cells. In contrast, no such staining of the nuclei of nondividing cancer cells was observed even when adjacent parenchymal cells were stained. In dividing cancer cells, the chromosomes sometimes stained with moderate intensity.

Tissue Smears

Fresh tissue smears.—Smears were made of liver, kidney, and spleen from control and tumor-bearing mice. Smears were also made of breast cancer cells of mice and from diverse cancerous and parenchymal cells of human beings. The smears were allowed to dry at room temperature and studied in relation to the influence of fixation and extraction on the staining with A-S.

Untreated smears.—Unfixed, air-dried smears of control kidney, spleen, liver, and breast cancer cells from mice, and homologous, diverse tissues of human beings failed to stain with A-S.

Formalin-fixed smears.—Air-dried smears were immersed in 10 percent neutral formalin for a minimum of 10 minutes. They were then rinsed 7 times with distilled water and stained with A-S. After the A-S staining, the kidney, liver, and spleen cells from mice exhibited a precise chromatin-type staining of the nuclei which resembled the staining seen in the tissue sections from mice or human beings responding to antigenic stimuli. The cytoplasm of the various cell types was unstained except for a minimal to moderate granular stain in the cytoplasm of some liver cells (fig. 8). A similar type of distinct staining of the nuclear chromatin was observed in smears of diverse types of noncancerous human parenchymal and stromal cells, *i.e.*, liver, kidney, spleen, gastric mucosa, small intestinal mucosa, colon mucosa (fig. 9), gallbladder mucosa, lymph nodes, acinar adenoma of the breast, polypoid adenoma of the colon, proliferative phase of the endometrium, uterine cervix, thyroid adenoma, esophogeal mucosa, bone marrow, and 4-month-old fetal tissues.

In contrast to the structured appearance of the noncancerous cells, the smears of intermitotic cancer cells of mice and human beings had diffuse granular staining of the nuclei, having minimal internal differentiation and appearing remarkably similar in various types of cancer cells, *viz.*, cancer of the breast in mice, human cancers of the breast, gallbladder, esophagus, colon, thyroid, stomach, melanoma, lung, endometrium, ovary,

lymphosarcoma, and acute leukemia (figs. 10 and 11). Similar staining was also observed in the nuclei of the HeLa strain of human epidermoid carcinoma grown in media supplied by Lederle Laboratories, consisting of 5 percent calf serum, 5 percent lactol in balanced salt solution. Cultures of C3H mouse fibroblasts and monkey kidney cells, which showed numerous mitotic figures and were grown in the same type of medium as the HeLa cells, stained with A-S like noncancerous cells. These latter findings, coupled with the observations on the staining of smears of tissues with high growth potential—the bone marrow, hyperplastic lymph nodes, fetal tissues, proliferative phase of the endometrium, basilar cells of the uterine cervix, polypoid adenoma of the colon, and fetal adenoma of thyroid—indicate that growth potential *per se* does not produce the changes in A-S staining of cell nuclei seen in cancer cells.

In cancer cells undergoing mitosis, the chromosomes were stained and appeared similar to the chromosomes of dividing noncancerous cells. However, on oil immersion the stained chromosomes of cancer cells appeared as condensations of extremely fine granules, whereas such granulations were not as apparent in the chromosomes of noncancerous cells.

Alcohol-fixed smears.—Smears of parenchymal and cancer cells of mice and human beings were fixed in 95 percent ethyl alcohol for 10 minutes. The slides were then washed with distilled water and "stained" with A-S. No staining was observed, nor was staining seen in slides fixed in absolute alcohol, or in an ether-alcohol fixative as used in the Papanicolaou techniques. Smears fixed in absolute alcohol, washed in distilled water, and fixed in 10 percent formalin for 10 minutes showed minimal staining with A-S.

Formalin-fixed smears exposed to alcohol.—Smears of parenchymal and carcinoma cells were fixed in 10 percent neutral formalin for 10 minutes. The smears were rinsed in 7 changes of distilled water and placed successively in Coplin jars containing 95 percent absolute alcohol and absolute ethyl alcohol. The exposure time in each alcohol was 5 minutes. The smears were rinsed with distilled water and "stained" with A-S. Smears treated in this fashion showed minimal staining of the nuclei. These results parallel the lack of staining seen in routine tissue sections. It is well known that in the routine preparation of paraffin sections the formalin-fixed tissues are similarly exposed to alcohol.

To gain further understanding of the A-S staining reactions, we investigated the influence of the following types of extraction on the staining of smears by A-S:

(*a*) *Saline extraction:* Air-dried smears of control and cancerous tissues were placed in 5° C. 0.9 percent saline, *p*H 7.2 for 15 minutes. This type of extraction tends to remove albumins and globulins. The smears were then washed with cold distilled water and fixed in 10 percent neutral formalin for 10 minutes before staining with A-S. This procedure caused no appreciable change in the staining of the nuclei as compared to unextracted smears.

Studies were also made of the effect of saline extraction of minced control and cancerous tissues. The minced fresh tissues were agitated in 10 times their volume of cold saline, buffered to *p*H 7.2 for 15 minutes. The suspension was centrifuged, the saline decanted, and the saline extraction repeated. After the second extraction, smears were made of the minced tissues, fixed in formalin, and stained with A-S. The type of staining was similar to that in unextracted cells.

(*b*) *Acid extraction:* Air-dried smears were extracted with saline as previously described and were further extracted with 5° C. 0.25 N HCl for 30 minutes. This type of extraction tends to remove histones from the cells. The smears were rinsed with distilled water, fixed in 10 percent neutral formalin for 10 minutes, and stained with A-S. Smears treated in this fashion lost their ability to stain with A-S. Scattered silver granules were found in the cytoplasm and extracellularly, but the structured staining of the nuclei was absent.

We have also extracted with acid minced control and cancerous tissues, with and without prior saline extraction. Smears were made of the cells after 30 minutes extraction with cold, 0.25 N HCl. The smears were fixed in formalin and stained with A-S. The nuclei of such smears showed minimal or no staining with A-S.

(*c*) *Ten percent saline, pH 7.2 extraction:* It is well known that concentrated saline solutions dissolve nucleoproteins. Air-dried smears of fresh liver cells were extracted for 30 minutes with 10 percent aqueous solutions of sodium chloride buffered to *p*H 7.2. After extraction, the smears were rinsed with distilled water, fixed in formalin, and stained with A-S. These smears showed minimal or no staining of their nuclei.

(*d*) *Feulgen-type hydrolysis:* Air-dried smears were incubated in 1 N HCl at 60° C. for 30 minutes. The smears were then washed in water, fixed in formalin as previously described, and stained with A-S. Smears treated in this fashion failed to stain with A-S.

(*e*) *Deoxyribonucleic acid (DNA) extraction:* Formalin-fixed smears were placed in boiling 5 percent trichloroacetic acid (TCA) for 15 minutes. The slides were run through alcohol and water to remove the TCA and placed in formalin for 10 minutes. They were then washed in water and stained with A-S. The nuclei were stained like the unextracted formalin-fixed smears. However, if the second formalin fixation was omitted, no A-S staining was obtained.

These observations suggested that A-S staining of cell nuclei depended upon the presence of formalin-fixed, acid-extractable nuclear proteins (AENP). To test this possibility further, we undertook the following experiment: The liver was removed from an adult CFW mouse and smears were prepared from a small sample. These smears were fixed in formalin and stained with A-S as previously described. The typically stained nuclei of these smears served as a control for the subsequent studies.

The main portion of the liver was sliced into minute fragments and was then extracted with 0.9 percent saline, *p*H 7.2 at 5° C. for 15 minutes, centrifuged, rinsed in cold, distilled water, and extracted with 15 ml. of

0.25 N HCl at 5° C. for 30 minutes. The acid-extracted liver suspension was centrifuged and the supernatant solution decanted and saved. The acid-extracted liver cells (AEL) were washed twice with cold HCl and then with distilled water. Smears of a sample of the AEL showed minimal or no A-S staining of the nuclei after formalin fixation (fig. 12). In contrast, smears of the saline-extracted tissues retained A-S staining of the nuclei. Both saline and acid-extracted cells retained their normal appearance and the nuclei were readily stained with methylene blue.

The acid extract was handled as follows: Concentrated ammonium hydroxide (2.5 ml.) was added which caused a precipitate to form. The solution stood at room temperature for 10 to 20 minutes and the suspension was then centrifuged and the supernatant fluid decanted. Excess 95 percent ethanol was added to the supernatant which caused additional precipitation. After standing 10 to 20 minutes at room temperature, the mixture was centrifuged and the supernatant decanted. We also obtained combined ammonia and ethanol precipitates by adding ethanol directly to the ammonia-treated acid extracts.

Incubation of AEL with the ammonia-precipitated, acid-extractable protein, or the ethanol-precipitated protein, or the combination of both precipitates suspended in distilled water, at slightly alkaline *p*H, restored the ability of the nuclei to stain with A-S (fig. 13). The precipitates themselves could be stained with A-S if they were first fixed in formalin. Unfixed or alcohol-fixed precipitates do not stain with A-S.

The ability of the acid-extracted proteins to restore the A-S staining to the acid-extracted nuclei is strong evidence that the A-S technique used in the present study is precise for visualizing formalin-fixed AENP. Similar results have also been obtained with spleen and cancerous tissue.

When liver cells were extracted with hot trichloroacetic acid after the cold acid extraction, DNA as well as AENP were removed. Such cells did not stain with A-S, nor did the incubation of these cells with the AENP extracts bring about a return of staining with A-S. The removal of the DNA apparently destroys the AENP binding of the nuclei and thus the A-S staining. The extraction of DNA was confirmed by the failure of the extracted nuclei to stain with methylene blue. Although methylene blue is not a specific stain for DNA, the lack of staining of the TCA-extracted cells is strong indication that DNA is absent or markedly reduced.

DISCUSSION

The data obtained in the present study may be summarized as follows:

1. A-S does not stain the nuclei of formalin-fixed, routinely prepared (formalin fixation, to dehydrating alcohols, xylol, paraffin xylol, descending series of alcohols, water, and silver stain) sections of tissues from human beings or mice free of systemic infections, antigenic stimulation, or self-perpetuating degenerative diseases.

2. A-S commonly stains the nuclei of routine formalin-fixed sections of noncancerous control, hyperplastic, and adenomatous tissues from human beings and mice having

certain infectious diseases, self-perpetuating degenerative diseases, or after exposure to antigens.

3. A-S does not stain the nuclei of intermitotic carcinoma cells in routine formalin-fixed sections of cancerous tissues from human beings or mice, whether or not the nuclei of the parenchymal cells were stained.

4. Neither fresh, unfixed nor alcohol-fixed smears of diverse tissues were stained with A-S.

5. The nuclei of formalin-fixed smears of parenchymal and stromal cells of human beings and mice were prominently stained by A-S.

6. Formalin-fixed smears of intermitotic carcinoma cells exhibited a characteristic type of A-S staining of the nuclei which was similar among cancer cells of diverse types but different from the staining of noncancerous, homologous cells.

7. A-S staining of formalin-fixed smears was decreased by exposure of the formalin-fixed smears to alcohol before exposure to A-S. *Note* that such exposure to alcohol after formalin fixation is routine in the processing of tissues for paraffin sections.

8. A-S staining of formalin-fixed smears was prevented by extraction with concentrated saline solutions prior to formalin fixation.

9. A-S staining of formalin-fixed smears was prevented by acid extraction prior to formalin fixation.

10. A-S stained formalin-fixed AENP fractions but did not stain unfixed or alcohol-fixed smears of the extracted proteins.

11. Incubation, at alkaline *p*H, of acid-extracted cells with AENP extracts restored the ability of the cell nuclei to be stained with A-S.

These data suggest that the A-S staining of formalin-fixed nuclei may be a precise type of chemical reaction rather than a nonspecific absorption phenomenon. Under the conditions used, A-S stained formalin-fixed AENP. The exact biochemical mechanism of this type of staining remains to be elucidated. However, the binding or reaction of AENP with formalin is a critical precursor to the silver staining of nuclear structures. The lack of A-S staining in routine tissue sections in contrast to the distinct straining of the nuclei of formalin-fixed smears seems to be due to the removal and/or inactivation of appreciable amounts of the bound formaldehyde during the preparation of the tissue section.

The observation that A-S stained the nuclei of tissue sections from patients with a variety of diseases suggests that the formalin binding of AENP may be appreciably increased in the presence of certain disease processes. Conversely, the lack of staining of the nuclei of sections of cancerous tissues, even when the noncancerous cells were stained, and the distinctive type of staining encountered in the nuclei of smears of cancerous cells indicates a significant, if not unique, structural and/or chemical change in the AENP of cancer cells.

We have also observed the staining of cell nuclei and isolated AENP with Fast Green according to the method of Alfert and Geschwind (7). These authors reported that at alkaline *p*H, Fast Green stains histones in a selective fashion. Fast Green stained our isolated AENP fractions intensely, which suggests that the silver-stainable AENP is, or includes appreciable amounts of, nuclear histone. Furthermore, the nuclei of acid-extracted cells did not stain with either Fast Green or A-S. The observation that ammonia caused precipitation of a fraction of the AENP and that the precipitate could be stained with both Fast Green and A-S also suggests that the AENP is rich in nucleohistone.

Staining with Fast Green did not prevent subsequent staining with A-S, nor did A-S staining prevent subsequent staining of the isolated fractions with Fast Green. It seems that Fast Green and A-S stain different reactive groups of AENP. This conclusion is supported by observations on the staining of smears. Fast Green did not stain intact, fresh, or formalin-fixed cells. To achieve staining of cell nuclei with Fast Green it was necessary to remove the DNA by hot trichloroacetic acid extraction. A-S staining does not require prior removal of DNA, but does depend on formalin fixation. Fast Green stains the DNA binding groups of the nuclear proteins while A-S reacts with non-DNA binding groups.

As was true of the isolated AENP, the nuclei of smears may be doubly stained. Such doubly stained nuclei of noncancerous cells clearly show that chromatin structures stained with A-S are also stained with Fast Green. However, not all Fast Green stained areas were necessarily stained with A-S. The Fast Green showed a diffuse and chromatin distribution of the stain, while the silver staining was limited almost exclusively to chromatin structures. The lack of complete coincidence between the Fast Green and A-S staining was more prominent in the cancerous cells than in the control cells. Since A-S stained intermitotic cancer cell nuclei in the form of diffusely scattered fine granules, the doubly stained cancer cell nuclei were characterized by a greater prominence of Fast Green staining of nuclear structures which were not also stained with A-S. In control and cancer cells undergoing mitosis, the chromosomes were stained by both A-S and Fast Green.

The data suggest that the observed differences in A-S staining of cancer cell nuclei compared with control cell nuclei might reflect differences in the nuclear histones of cancer cells, though other possiblities have not been rigorously excluded. An examination of the literature concerning the histones of cancer cells does not reveal unanimous opinion. The studies of Cruft *et al.* led them to conclude that histones tend to be cell-specific both in normal cells and in cancer cells, that the histones of cancer cells differ from the histones of homologous control cells in regard to solubility and electrophoretic mobility (*8*), and that the nuclei of malignant cells contain smaller amount of histones than homologous normal cells. On the other hand, Busch and Davis, in a review of the nuclear proteins of tumors and other tissues, did not feel that clear-cut differences had been demonstrated between control and cancer cell histones (*9*). These authors seemed more inclined to the view of Davison: "It is possible that the malignant transformation is accompanied by an abnormal histone, but the latter will apparently not be revealed by a study of the gross characteristics of the histones. It will require a more complete resolution of the individual components of the histone mixture than has yet been achieved" (*10*).

The significance of the histones in cancer cells is also indicated by the recent report of Busch *et al.* on the amino acid incorporation into nuclear proteins of rat tumors (*11*). They observed that the Walker tumor in-

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corporated 12 of 17 amino acids into histone to a greater extent than into the protein of the whole homogenate. In contrast, the nonneoplastic tissues showed a preferential incorporation of the amino acids into cytoplasmic proteins. The authors suggested that tumors might be considered as a tissue specialized toward nuclear protein synthesis, particularly histones. Recently Davis and Busch have used a combination of chromatography and radioactive tagging techniques to analyze nuclear proteins (*12*). They demonstrated a high degree of specificity of the chromatographic patterns of the histones from different tissues. In addition, they observed that approximately one third of the radioactive lysine incorporated into tumor histone was in a fraction which was not seen in the nontumorous tissues studied.

These data indicate the necessity of knowing more about the histones and AENP of cancer cells. This study, which was initiated by an inquiry into what appeared to be vagaries of an emperic and capricious histologic stain, has led to a most intriguing sequence of observations: (*a*) Various types of cancer cells were stained in a similar fashion by A-S; (*b*) the staining of the cancer cells studied differed from that of noncancerous cells of diverse types; (*c*) under the conditions of study, A-S stained AENP of control and cancer cells; (*d*) silver stainable AENP had histone-like properties.

However, it is not known whether (*a*) alterations in AENP are universal in, and specific for, cancer cells; (*b*) AENP is histone *in toto* or in part; (*c*) the observed changes in cancer-cell AENP are due to intrinsic changes in molecular species, differences in physical state of aggregation, or the presence of absorbed "foreign" protein or virus.

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PLATE 136

FIGURE 1.—Liver section, CFW control mouse, A-S staining. *Note* lack of distinct staining of nuclei.

FIGURE 2.—Kidney section, CFW control mouse, A-S stain. *Note* lack of distinct staining of parenchymal cells. There is some staining of nuclei of scattered lymphoid cells.

PLATE 137

FIGURE 3.—Liver section, CFW mouse, 24 hours after injection of diphtheria toxoid; A-S stain.

FIGURE 4.—Kidney section, same mouse as in figure 3.

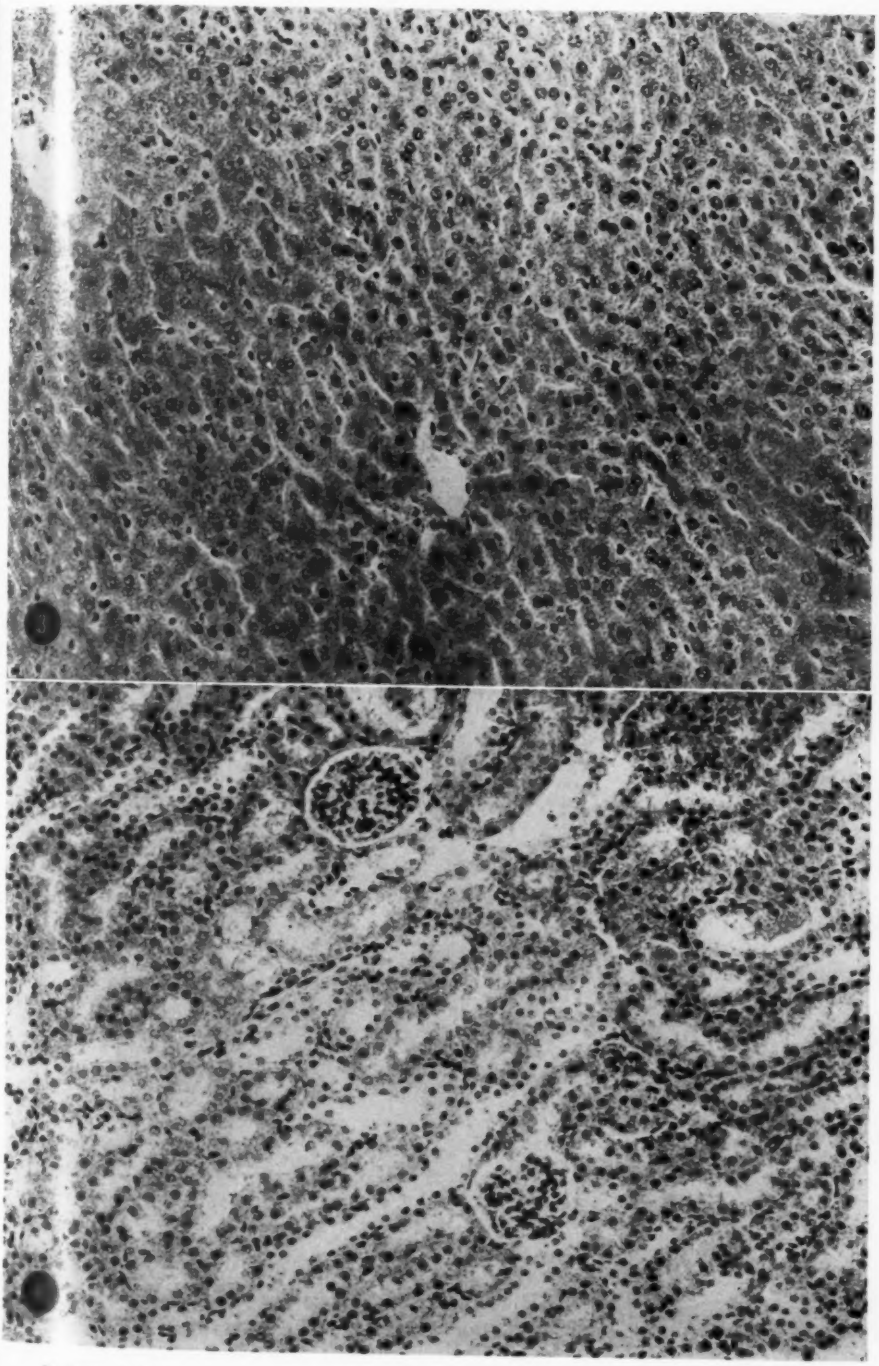


PLATE 138

FIGURE 5.—Section of human kidney biopsy (4573-58), subacute glomerulonephritis. *Note* distinct staining of nuclei of glomerules and somewhat less intense staining of nuclei of tubular cells.

FIGURE 6.—Section of human breast, biopsy (4199-58), lobular carcinoma *in situ* adjacent to area of adenosis, A-S stain. *Note* difference in staining between nuclei of cancerous and noncancerous cells.

FIGURE 7.—Section of human kidney, autopsy, (FM-1452), lupus erythematosis disseminata, A-S stain. *Note* prominent staining of nuclei of glomerular and tubular cells.

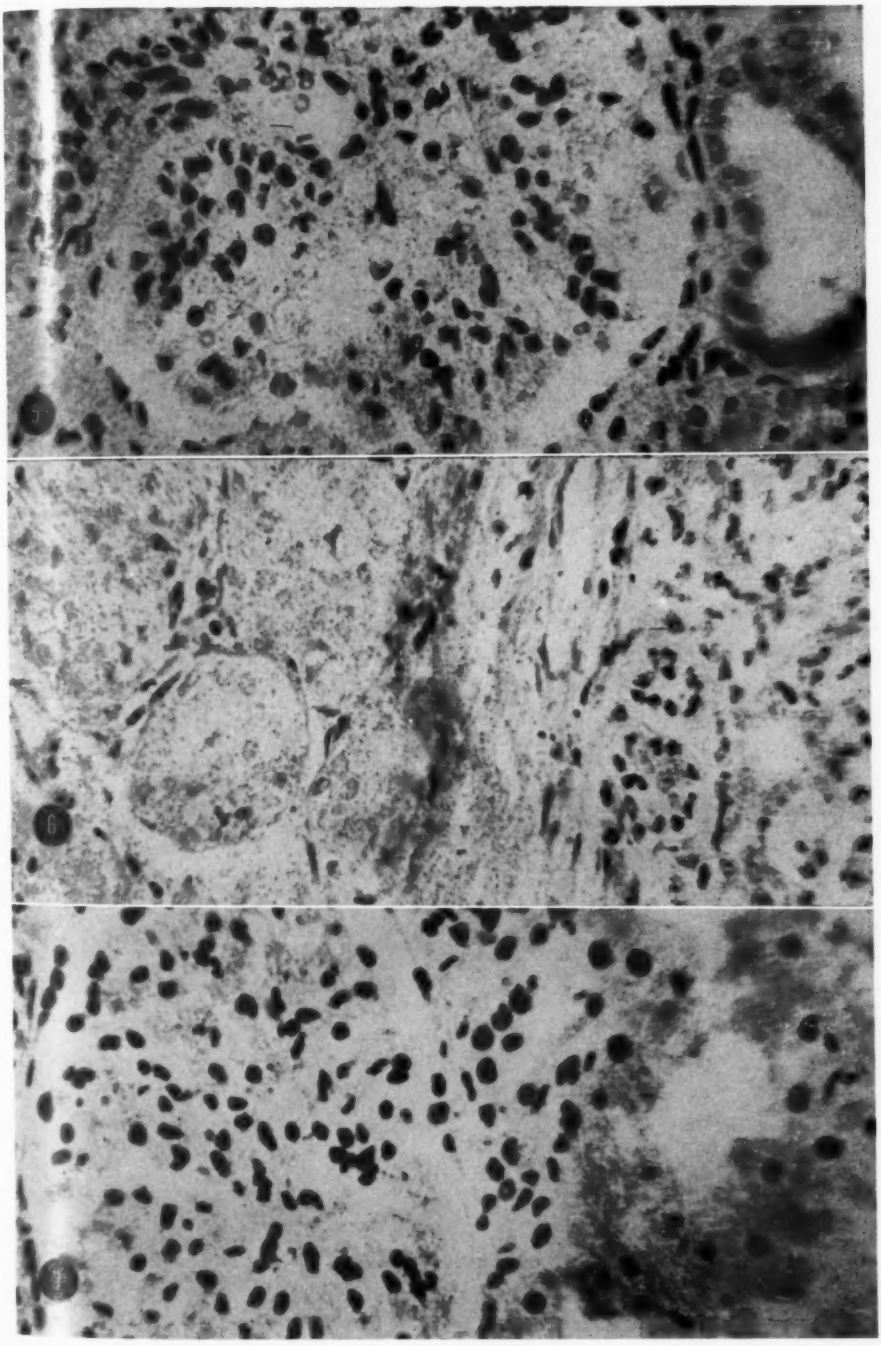


PLATE 139

FIGURE 8.—Smear, mouse liver, formalin-fixed, A-S stain. *Note* distinct chromatin pattern in all nuclei.

FIGURE 9.—Smear, human colon mucosa (6260-59), formalin-fixed, A-S stain. *Compare* distinct "chromatin" pattern with punctate granulations of cancer cells from same patient, figure 10.

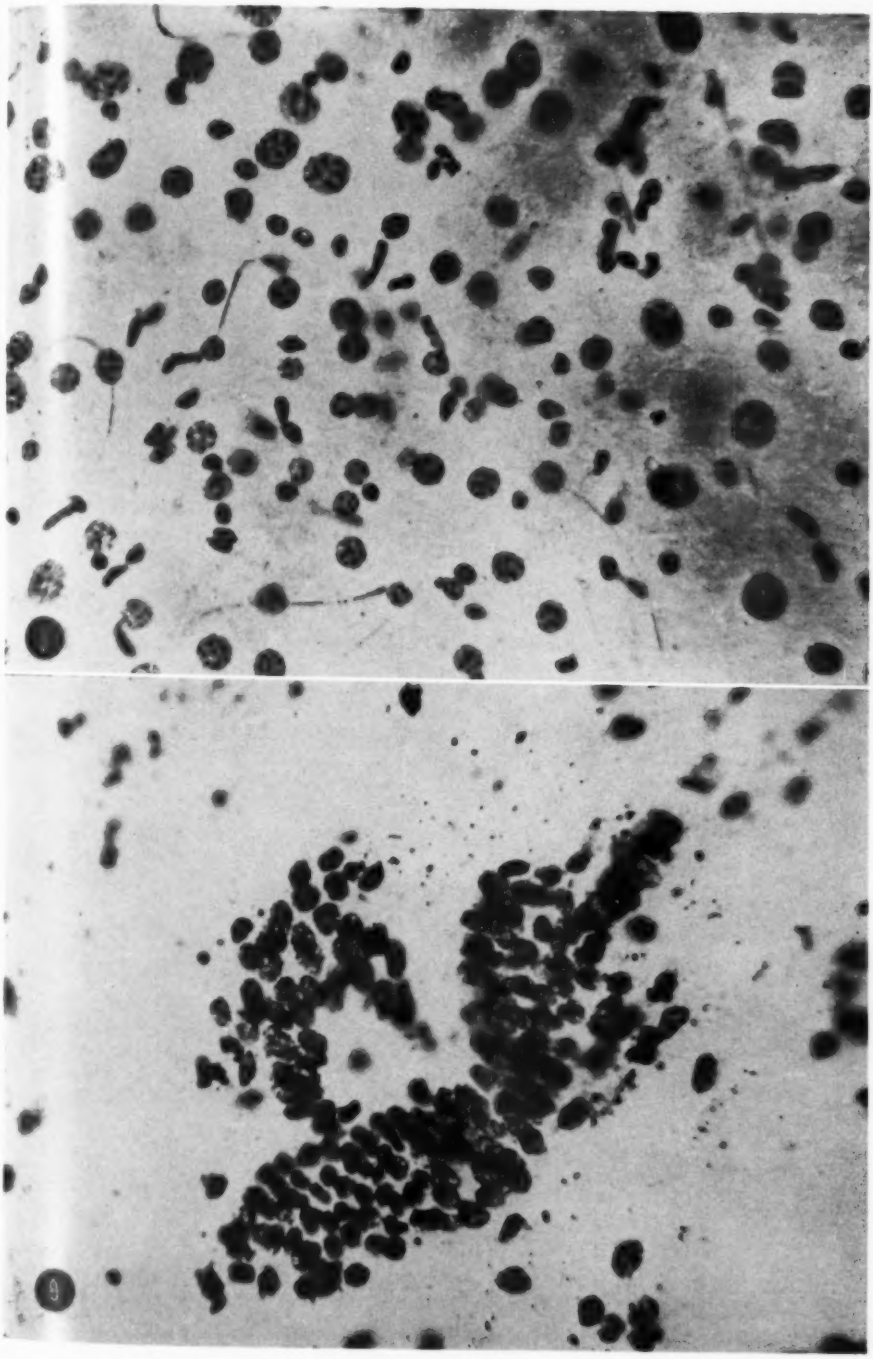


PLATE 140

FIGURE 10.—Smear, human colon carcinoma (6260-59), formalin-fixed, A-S stain. *Note* fine punctate granulation in nuclei of cancer cells. Nuclei of scattered inflammatory cells show dense staining.

FIGURE 11.—Smear, human lymph node, acute leukemia, autopsy specimen, formalin-fixed, A-S stain. *Note* sparce punctate staining of blast cells in contrast with "chromatin" type staining of lymphocytes.

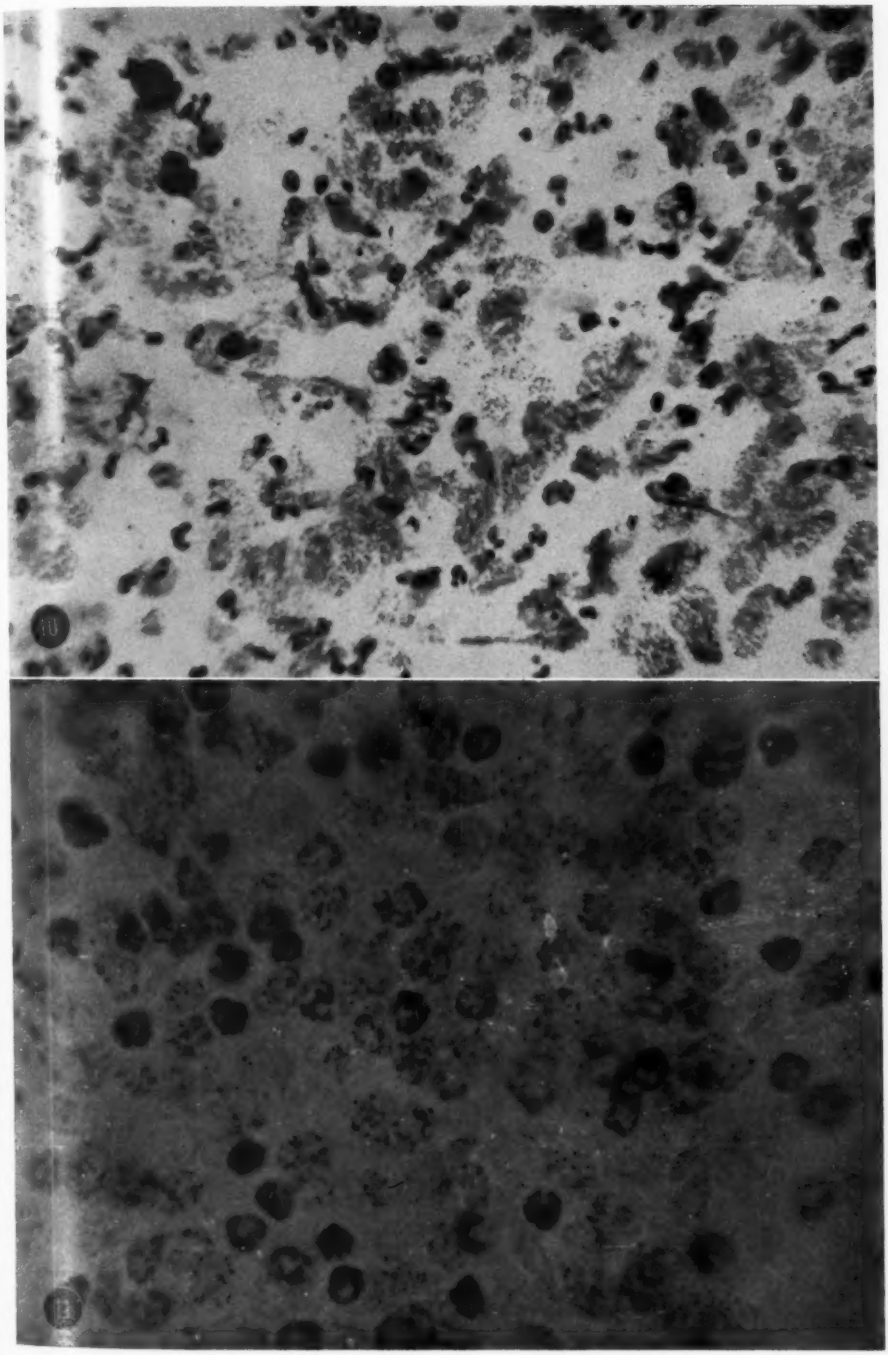
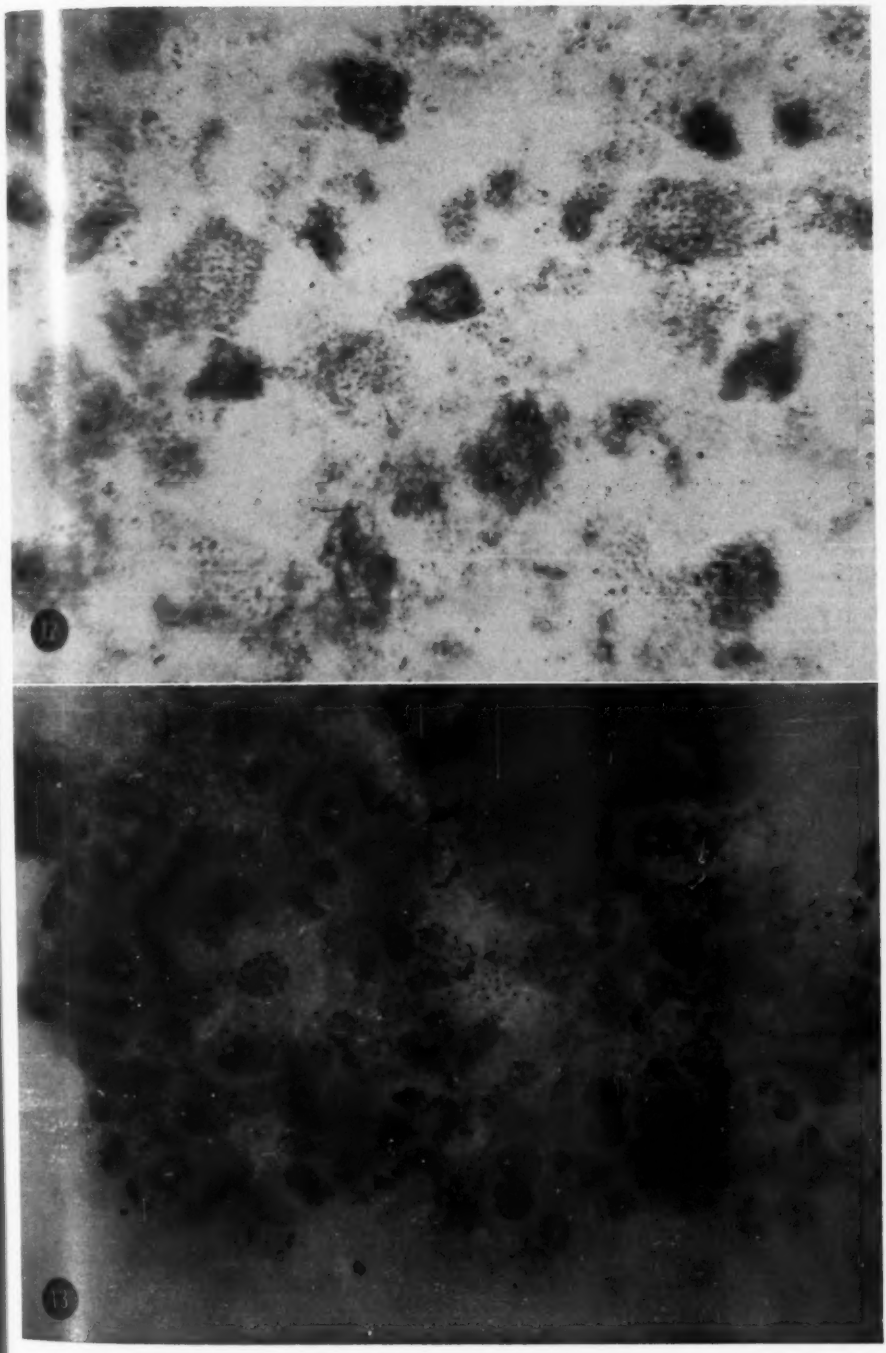


PLATE 141

FIGURE 12.—Smear, acid-extracted mouse liver, formalin-fixed, A-S stain. *Note* lack of staining of nuclei.

FIGURE 13.—Smear, acid-extracted mouse liver (same preparation as in fig. 12) incubated with AENP, formalin-fixed, A-S stain. *Note* return of staining of nuclei.



Mammary Carcinogenesis by 3-Methylcholanthrene. II. Inhibitory Effect of Pregnancy and Lactation on Tumor Induction [1,2]

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SUMMARY

These experiments are compatible with our earlier postulation that in mammary carcinogenesis a two-stage mechanism exists: the initiating process induced by the action of carcinogen, and the promoting phase regulated by the hormones. Hormonal stimulation is ineffective in accelerating the appearance of a tumor in the absence of "latent" tumor cells. Our data further showed that hormones of pregnancy and lactation, rather than a direct local effect of lactation, are important factors in the inhibition of mammary carcinogenesis by 3-methylcholanthrene since the prevention of suckling in pregnant rats did not increase tumor incidence. Chemical determinations of the level of 3-methylcholanthrene in mammary tissues indicated that the clearance of the carcinogen from the mammary tissues was greatly accelerated in pregnant and lactating rats. It would therefore appear that two factors might be important: First, the accelerated rate of 3-methylcholanthrene metabolism in pregnant and lactating rats prevented concentration of the carcinogen in the target tissue, and, second, the abnormal functional state of the mammary gland produced by the excessive hormones elaborated during pregnancy. The data suggested that the critical requirements for the induction of mammary cancer by 3-methylcholanthrene are dependent upon (*a*) an adequate time for the initiating process, (*b*) the concentration and persistence of the carcinogen in the target tissue for its effect, and (*c*) the functional state of the mammary gland exposed to the carcinogen.—J. Nat. Cancer Inst. 25: 991–1003, 1960.

WHEN 3-METHYLCHOLANTHRENE was fed in multiple doses to immature or young adult female Sprague-Dawley rats, which subsequently became pregnant, mammary cancer appeared early and the rate of tumor growth was often accelerated. The cancer regressed after parturition, but growth was accelerated when rats became pregnant again (*1*). The enhancing effect of pregnancy on tumor development was abolished, however, if the carcinogen was fed to the already pregnant or lactating rats. In this instance, pregnancy obviously inhibited tumor induction.

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[2] This study was supported by grant C-4632 from the National Cancer Institute, National Institutes of Health, Public Health Service.

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The present study was undertaken to determine whether the inhibitory effect of pregnancy and lactation in tumor induction was the direct effect of lactation on the mammary gland or the result of the hormonal changes in pregnancy or lactation. The results of the experiments described in this paper indicate that the hormonal changes in pregnancy and lactation, rather than a direct local effect of lactation, are important factors in the inhibition of mammary carinogenesis. In addition, our data suggest that the critical requirements for the induction of mammary cancer by 3-methylcholanthrene are dependent upon: (*a*) an adequate time for the initiating process, (*b*) the concentration and persistence of the carcinogen in the target tissue for its effect, and (*c*) the functional state of the mammary gland exposed to the carcinogen.

MATERIALS AND METHODS

In all experiments, 55- to 60-day-old mature female Sprague-Dawley albino rats were used. 3-Methylcholanthrene, dissolved in sesame oil in a concentration of 10 mg. per ml., was fed daily, 6 days a week, through a stomach tube.

In the first experiment, the rats were fed 10 mg. of 3-methylcholanthrene daily, for 6, 10, and 15 days. These rats were subsequently mated either immediately after the completion of feeding or 5 to 10 days later.

In experiments on the effect of pregnancy on mammary-tumor incidence, rats were given only a single dose of 30 mg. of 3-methylcholanthrene in 1 ml. of sesame oil and then were mated at intervals of 10, 15, 20, 25, and 30 days.

In the study of induction of mammary cancer during pregnancy, female and male rats were housed in the same cage for 7 days and pregnancy was ascertained. The pregnant rats were divided into two groups: one group receiving 10 mg. of 3-methylcholanthrene for 6 days and the other, for 10 days. Both groups were further divided into 3 subgroups: (1) rats which had normal parturition, (2) rats in which cesarean sections were performed about 15 days after mating, and (3) rats in which all placentas were retained at the time of cesarean section. A control group of virgin rats was given 3-methylcholanthrene for a similar length of time.

In another experiment, pregnant rats were fed 10 mg. per ml. of 3-methylcholanthrene daily for 10 days. At the end of the feeding, the animals were divided into two groups: In one, nipples on both sides were excised; in the other, only unilateral nipple excision was performed.

In the experiment on the effect of lactation on tumor induction, the lactating rats were fed 10 mg. of 3-methylcholanthrene daily for 15 days beginning on the 6th day after parturition.

To study the effect of 3-methylcholanthrene in an aqueous suspension, 55- to 60-day-old virgin rats were fed an emulsion of which 1 ml. contained 10 or 100 mg. of the carcinogen. One group of rats was fed the suspension containing 10 mg., daily, for 20 days; the other group received 100 mg.

of 3-methylcholanthrene in 1 ml. of suspension once a week for 3 weeks. The control group consisted of rats fed the usual sesame-oil preparation containing 10 mg. of 3-methylcholanthrene daily for 20 days.

The 3-methylcholanthrene emulsion was prepared as follows: A solution of 100 mg. of 3-methylcholanthrene in about 6 ml. of acetone was added to a boiling solution of 250 mg. Aerosol (sodium dioctyl sulfosuccinate, a nontoxic emulsifying agent manufactured by American Cyanamid Co.) in 10 ml. of water. The final concentration was 10 mg. of 3-methylcholanthrene per ml. A suspension containing 100 mg. per ml. was similarly prepared.

The rats were examined at frequent intervals for palpable tumors. We defined "latent period of tumor appearance" as the interval between the first feeding with 3-methylcholanthrene and detection of the first palpable tumor. All animals were killed at the end of 5 to 6 months. The mammary glands were examined carefully for any tumor at the time of necropsy. Organs suspected of containing metastases were removed for histological sections.

In experiments involving concentration and clearance of 3-methylcholanthrene in mammary glands, a single feeding of 30 mg. of 3-methylcholanthrene was given to 3 groups of 30 rats each: virgin, pregnant, and lactating. Five animals from each group were killed 1, 2, 3, 4, 7, and 10 days after feeding. The breast and fatty tissues from retroperitoneal regions and the abdominal cavity were dissected out and the milk curd was removed from the stomachs of the nursing infant rats for determination of concentration of 3-methylcholanthrene in these tissues and in the milk. The breast was separated from the surrounding adipose tissues by careful dissection. Identical experiments were also made, with an aqueous suspension prepared as described. Adult virgin female rats were given a single feeding of 1 ml. of the suspension containing 100 mg. of 3-methylcholanthrene and then killed 1, 2, 3, 4, 7, and 10 days after feeding. The method of extraction and final assay of 3-methylcholanthrene with the Aminco-Bowman spectrophotofluorometer has been described previously by Dao *et al.* (*2*).

RESULTS

Incidence of Distant Metastases in Rats Bearing Induced Mammary Cancer

Although metastases to the regional lymph nodes have been observed in many instances, distant metastases are not common. Of 320 rats necropsied in this study so far, distant metastases to the lungs were observed in only 2 rats (figs. 1 and 2).

Effect of Pregnancy on Tumor Induction

The results shown in table 1 again demonstrated that pregnancy shortened the latent period of tumor appearance, which was practically the

same (all appeared before 50 days) in all pregnant groups, irrespective of the total dose of carcinogen given. The difference in time of appearance of palpable tumors between the control and pregnant groups was statistically significant ($P<0.01$). The incidence of mammary cancer in the pregnant and control groups, however, appeared to depend on the amount of carcinogen given.

TABLE 1.—Effect of pregnancy and duration of administration of 3-methylcholanthrene on induction of mammary tumors*

Group	Number of rats	Duration of 3 MCA administration (*days*)	Rats with tumors		Appearance of palpable tumors (*days*)		
			Number	Percent	Range	Median	Mean†
Control	33	6	21	64	44–162	93	107 ± 38
Pregnancy‡	10	6	0	0	—	—	—
Pregnancy§	15	6	8	53	42–48	43	44 ± 2
Control	28	10	26	93	43–153	90	95 ± 34
Pregnancy‖	10	10	8	80	37–47	39	41 ± 3
Control	35	15	34	97	45–130	59	68 ± 22
Pregnancy‖	18	15	15	83	36–47	38	40 ± 3

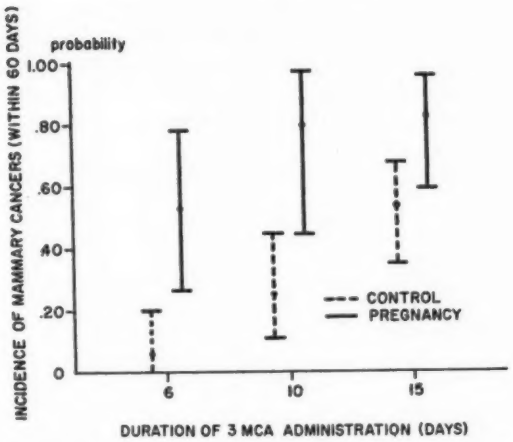
*All animals were observed for 6 months.
† ± Standard deviations.
‡Rats were mated immediately after the last oral feeding of 3-methylcholanthrene.
§Rats were mated 10 days after the last oral feeding of 3-methylcholanthrene.
‖Rats were mated 5 days after the last oral feeding of 3-methylcholanthrene.

When the incidence of mammary cancer observed within 60 days in control and pregnant groups was compared, the difference became even more striking (table 2). The dose response was also clearly demonstrated (text-fig. 1). When long-term observation was made, the number of rats developing mammary tumors continued to rise in the control group, while the number in the pregnant group remained the same. In the latter group, if tumors developed they appeared some time before parturition, regardless of the total dose of carcinogen given, or else they did not develop at all. These observations indicate that the promoting agent, in this case the hormones elaborated during pregnancy, while capable of accelerating the tumor appearance, might also inhibit the tumor development.

TABLE 2.—Effect of pregnancy on rapid induction of mammary cancers—incidence of mammary cancers appearing within 60 days after first feeding of 3-methylcholanthrene

Group	Number of rats	Duration of 3-MCA administration (*days*)	Rats with tumors	
			Number	Percent
Control	33	6	2	6
Pregnancy*	15	6	8	53
Control	28	10	7	25
Pregnancy†	10	10	8	80
Control	35	15	19	54
Pregnancy†	18	15	15	83

*Rats were mated 10 days after the last oral feeding of 3-methylcholanthrene.
†Rats were mated 5 days after the last oral feeding of 3-methylcholanthrene.



TEXT-FIGURE 1.—Graph illustrates effect of pregnancy and dose of 3-methylcholanthrene on rapid induction of mammary tumors. The difference in incidence of mammary cancer between control and pregnant groups is significant at a 5 percent level by the chi-square test in 6 and 10 days regimen.

When rats received a single dose of 3-methylcholanthrene (30 mg./ml. in sesame oil), 60 percent developed mammary cancer between 50 and 137 days. But when they were mated after a single feeding of the carcinogen, the incidence of cancer rose as the interval between the time of feeding and subsequent pregnancy lengthened (table 3). This experiment clearly demonstrated the critical significance of time between initiation and promotion in tumor induction. In these pregnant groups, if tumors developed, they appeared some time before parturition.

TABLE 3.—Influence of pregnancy on incidence of mammary tumors in rats*

Group	Interval† (*days*)	Number of rats	Rats with tumors		Appearance of palpable tumors (*days*)		
			Number	Percent	Range	Median	Mean
Control	—	20	12	60	50–137	125	110
Pregnancy	10	32	3	9	30–45	45	40
Pregnancy	15	32	4	12	33–45	44	41
Pregnancy	20	29	7	24	35–44	40	39
Pregnancy	25	23	8	35	37–45	39	40
Pregnancy	30	10	5	50	35–47	39	42

*All animals received a single feeding of 30 mg. of 3-methylcholanthrene in 1 cc. sesame oil, and were observed for 5 months.

†Interval between feeding 3-methylcholanthrene and mating.

Induction of Mammary Cancer During Pregnancy

The incidence of mammary cancer was reduced from 63 to 20 percent, and from 93 to 36 percent on the 6- and 10-day regimens, respectively, when 3-methylcholanthrene was fed during pregnancy, irrespective of the

duration of carcinogen administration. When cesarean section was performed before parturition so that full lactation and suckling were prevented, no change in incidence of cancer was observed (table 4). Cesarean section with retained placentas before parturition yielded a tumor incidence much the same as that observed in cesarean section.

TABLE 4.—Induction of mammary cancer in pregnant rats*

Group	Number of rats	Duration of 3 MCA administration (*days*)	Rats with tumors		Appearance of palpable tumors (*days*)		
			Number	Percent	Range	Median	Mean
Control†	33	6	21	63	44–162	93	106
Normal parturition	35	6	7	20	92–178	104	127
Cesarean section	15	6	5	30	65–145	96	108
Control†	28	10	26	93	43–153	90	92
Normal parturition	25	10	9	36	85–148	96	106
Cesarean section	22	10	8	36	66–159	114	111
Cesarean section + placenta	12	10	5	41	65–140	90	98

*Fifty-five-day-old, Sprague-Dawley, mature female rats, were fed 3-methylcholanthrene during pregnancy. All rats were observed for 5 months.
†Nonpregnant rats.

Effect on Tumor Incidence of Unilateral or Bilateral Excision of Nipples in Pregnant Rats

In the group of rats whose nipples were excised bilaterally after administration of 3-methylcholanthrene and before parturition to prevent lactation, one tumor developed in 110 days. In the unilateral nipple-excision group, there was only one tumor, which appeared on the side where the nipples were not removed (table 5).

TABLE 5.—Effect of nipple excision on incidence of breast cancer in pregnant rats treated with 3-methylcholanthrene*

Group	Number of rats	Duration of 3-MCA administration (*days*)	Number of rats with tumors	Appearance of palpable tumors (*days*)
Normal parturition†	10	10	2	95, 125
Unilateral excision‡	10	10	1	86
Bilateral excision‡	10	10	1	110

*All animals were observed for 5 months.
†Without nipple excision.
‡Nipples excised on the last day of 3-MCA administration. All animals subsequently had normal parturition.

Induction of Tumor in Lactating Rats

Tumor induction was not observed in any of the 20 rats that received 3-methylcholanthrene during lactation.

Effect of 3-Methylcholanthrene in an Aqueous Suspension on the Induction of Mammary Cancer

When rats were fed the sesame-oil preparation of 10 mg. of 3-methylcholanthrene daily for 20 days, all developed mammary cancer between

49 and 120 days. No tumors were observed, however, in rats receiving the same dose of 3-methylcholanthrene in an aqueous suspension or in those given a dose of 100 mg. per ml. of aqueous suspension once a week for 3 weeks (table 6).

TABLE 6.—Effect of 3-methylcholanthrene in an aqueous suspension in the induction of mammary cancer in rats*

Group	Number of rats	Duration of administration (*days*)	Number of rats with tumors	Time of appearance of tumors	
				Range	Mean
Control†	10	20	10	49–120	75
Group 1‡	10	20	—	—	—
Group 2§	10	—	—	—	—

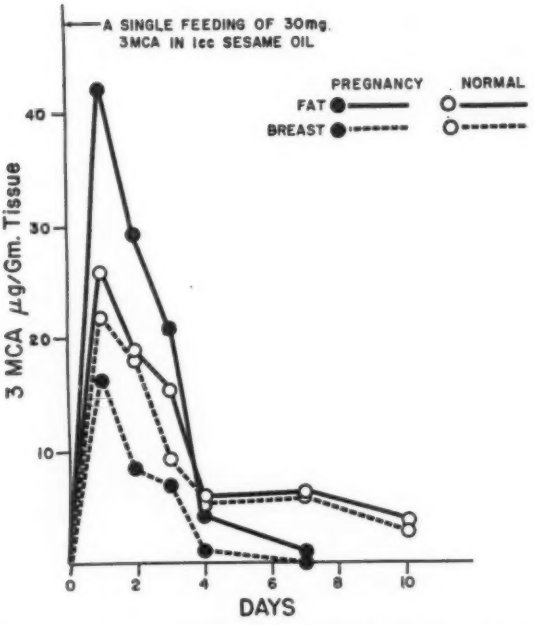
*All animals were observed for a period of 5 months.
† Received 10 mg. of 3-methylcholanthrene in 1 cc. of sesame oil.
‡Group 1 received 10 mg. of 3-methylcholanthrene in 1 cc. of aqueous suspension.
§Group 2 received 100 mg. of 3-methylcholanthrene in 1 cc. of aqueous suspension once a week for 3 weeks.

Clearance of 3-Methylcholanthrene in Breast Tissue of Virgin, Pregnant, and Lactating Rats

The results showed that 24 hours after oral administration of 3-methylcholanthrene to normal nonpregnant female rats, the concentration of the aromatic hydrocarbon rose to a high level in fat and breast tissue. It decreased rapidly within 72 hours, but a significant amount of 3-methylcholanthrene still remained in both tissues at the end of 7 days. In pregnant rats, the rate of clearance of 3-methylcholanthrene in fat and breast was rapid and no carcinogen was detected on the 7th day. The levels of 3-methylcholanthrene in fat and breast tissue were significantly different, *i.e.*, high in fat and low in breast, throughout the experimental period (text-fig. 2).

The clearance of 3-methylcholanthrene from the breast of lactating rats was even more rapid. Within 24 hours the carcinogen was rapidly excreted in the milk, as shown by the high concentration of 3-methylcholanthrene in the milk curd of the suckling newborn rats. Again no carcinogen was detected on the 7th day after the oral ingestion of 3-methylcholanthrene. The levels of 3-methylcholanthrene in fat and breast tissue were markedly different, significantly higher in the former but low in the latter. The levels of carcinogen in breast and milk curd were approximately the same (text-fig. 3).

When an aqueous suspension of 3-methylcholanthrene was given to the rats, the carcinogen failed to concentrate in either the breast or the fat. By the 3d day after a single feeding of 100 mg. of 3-methylcholanthrene in 1 ml. of aqueous suspension, no carcinogen was detected in either of these tissues (text-fig. 4).

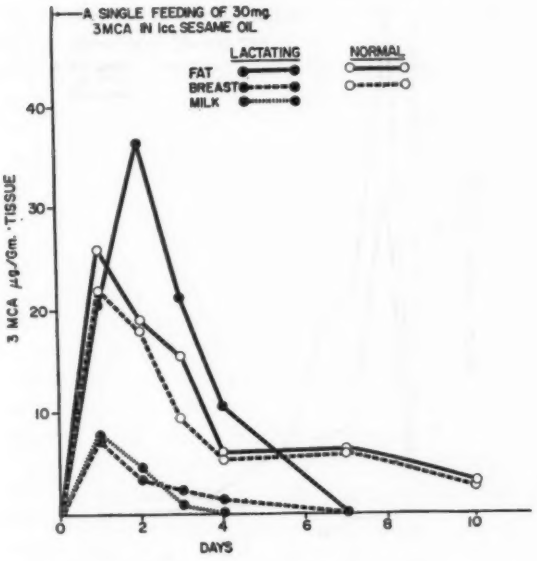


TEXT-FIGURE 2.—Clearance of 3-methylcholanthrene from the breast and fat after a single feeding of 30 mg. of the carcinogen to female virgin and pregnant rats. *Note* the rapid clearance of 3-methylcholanthrene from both breast and fat in the pregnant rats. While no 3-methylcholanthrene was found on the 7th day in the pregnant rats, significant and measurable amounts of carcinogen were still present on the 10th day in nonpregnant rats.

DISCUSSION

From the results of the present experiments in which virgin rats received a single feeding of 3-methylcholanthrene, it was evident that mammary cancer invariably developed after a long latent period. This latent period was shortened, however, if rats became pregnant. Furthermore, as the interval between the feeding of carcinogen and mating lengthened, the tumor incidence rose. That the tumors did not develop before 30 days in our experiments, irrespective of the dose of 3-methylcholanthrene and time of pregnancy, was evidence that a definite period was needed for the neoplastic transformation initiated by the carcinogen. This is compatible with our earlier postulation (*1*) that in mammary carcinogenesis a two-stage mechanism exists: the initiating stage induced by the action of the carcinogen and the promoting stage regulated by hormones.

In our experiments, the two-stage mechanism seemed to operate in two ways, both having the same initiating agent, the polycyclic hydrocarbon. The promoting agent, the hormonal stimulus, however, was introduced into two different situations: (*a*) in an endocrine state existing

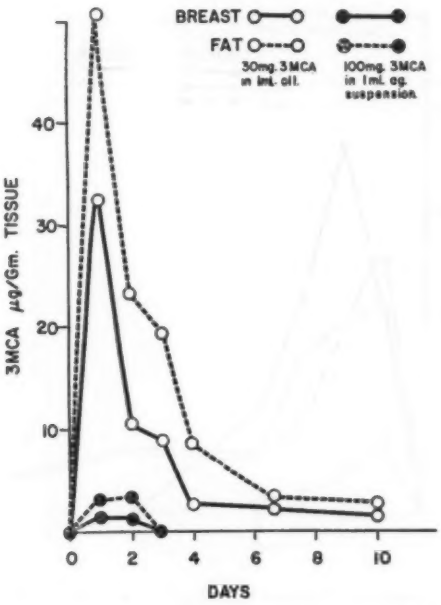


TEXT-FIGURE 3.—Clearance of 3-methylcholanthrene from the breast and fat after a single feeding of 30 mg. of the carcinogen in lactating and virgin female rats. The carcinogen was removed rapidly from the breast tissue in lactating rats. *Note* the high concentration of 3-methylcholanthrene in milk curd of the nursing newborn rats.

in the absence of pregnancy but in which ovarian hormones are essential, as shown by the development of tumors in mature female rats after administration of 3-methylcholanthrene, and the failure of tumor induction in male rats of the same strain (in male rats, 3-methylcholanthrene concentrates in breast tissue essentially the same as in virgin female rats); and (*b*) in the state of multiple hormonal stimulation induced by pregnancy in which hormones from pituitary and adrenal glands, ovaries, and placentas are elaborated. These hormones of pregnancy accelerate tumor development.

Although the precise nature of initiation and promotion is not yet fully elucidated, it does not seem probable that these two processes occur simultaneously, since hormonal stimulation is ineffective in accelerating the appearance of tumors in the absence of the "latent" tumor cells. Our experiments described so far support this view.

Earlier, Marchant (*3*, *4*) observed that the incidence of chemically induced mammary tumors in the milk-factor-free breeding IF strain of mice was reduced significantly (75–29%), if the mice were allowed to lactate fully. The author further demonstrated that repeated lactation was the essential factor in the lowering of the incidence of tumor induction. Marchant (*5*) attributed the inhibitory effect of lactation in the chemical induction of mammary cancer to a direct local effect of suckling on the breast. In our experiments, when rats were prevented from



TEXT-FIGURE 4.—Clearance of 3-methylcholanthrene from breast and fat after a single feeding of 100 mg. of the carcinogen in an aqueous suspension. *Note* the failure of 3-methylcholanthrene to concentrate in the breast and the fat when the carcinogen was given in an aqueous suspension. 3-Methylcholanthrene was still in both breast and fat in significant and measurable amounts on the 7th and 10th days, respectively, in rats receiving the carcinogen in sesame oil.

lactation by cesarean section before parturition, the tumor incidence remained the same as that in animals in which full lactation and suckling were allowed. If the inhibition of tumor appearance was due to a direct local effect of suckling in lactating animals, then the incidence of mammary cancer in animals which have never lactated should be the same as that in the control. This indeed is not the case. Furthermore, when the nipples of the rats were excised before parturition, there was again no rise in the incidence of mammary cancer.

Although the removal of the young and the excision of the nipples would prevent suckling and thus lactation in a broad sense, this would not prevent the mammary gland from filling up with milk, especially when cesarean section was done just before parturition. Thus our data suggest that hormones elaborated during pregnancy and perhaps lactation are the major contributing factors in the inhibition of mammary carcinogenesis by 3-methylcholanthrene.

The inhibitory effect of hormones of pregnancy and lactation on induction of mammary cancer cannot be readily explained. From the present work, it would appear that two factors may be important: first, the altered rate of 3-methylcholanthrene metabolism in pregnant and lactating

rats; and second, the abnormal functional state of the mammary gland produced by pregnancy and lactation.

Our earlier work (*2*) demonstrated that after the oral ingestion of 3-methylcholanthrene, the carcinogen concentrated mainly in the breast and fat of virgin female rats and in the milk and breast of lactating rats. The level of 3-methylcholanthrene in other tissues was either very small or insignificant. Further, the study indicated that the concentration and persistence of the carcinogen in the target tissues were important in chemical carcinogenesis. When 3-methylcholanthrene failed to concentrate in the mammary gland, mammary cancer did not develop. This was clearly shown by the fact that when an aqueous suspension of 3-methylcholanthrene was fed the rats, even in a large dose (100 mg.), the carcinogen failed to concentrate in the breast tissue, and mammary cancer never developed in these animals. The present data show that 3-methylcholanthrene was removed rapidly from the breast tissue and fat of pregnant rats after intragastric instillation of the carcinogen. Although 3-methylcholanthrene was present for long periods in the breast and fat of virgin female rats, its concentration was low and its clearance from these tissues in pregnant rats was rapid.

The clearance of 3-methylcholanthrene from the breast of lactating rats was even more rapid. The failure of 3-methylcholanthrene to concentrate as markedly in the mammary gland of pregnant rats and its rapid excretion through the milk after parturition are contributing factors in the reduction of tumor incidence. With radioactive methylcholanthrene, Shay *et al.* (*6*) showed earlier that methylcholanthrene can be transferred intact to the offspring by way of the milk. It is a distinct possibility that hormones may influence the rate of 3-methylcholanthrene metabolism.

The functional state of the mammary gland is also of critical significance in mammary carcinogenesis by 3-methylcholanthrene. While cancer of the breast did not form in atrophic mammary glands of hypophysectomized rats as reported earlier (*1*, *7*), the incidence of cancer was also reduced in rats with profound mammary hyperplasia. In the latter, the non-neoplastic proliferation inhibited the neoplastic transformation of the mammary tissues by 3-methylcholanthrene.

Huggins *et al.* (*7*) showed that the gestational or lactational type of mammary hyperplasia can be induced by the administration of an appropriate amount of equine gonadotrophin. The incidence of tumors in these rats so treated was similarly reduced. It is also true that steroid hormones such as estradiol-17β in large doses (10 μg.), although causing exuberant mammary growth, inhibit mammary carcinogenesis. The exact mechanism of such inhibition is yet to be clarified. The results in this paper show that augmentation of tumor growth can be obtained by increased hormonal stimulation as during pregnancy, but that such effect is nullified when the initiating and promoting processes are reversed in sequence. In fact, inhibition of tumor induction was possible, even during the initiating phase, by changing the target tissue from a "hormone-

appropriate" environment to an adverse endocrine condition, namely, the "hormone-excessive" environment occurring during pregnancy or as a result of hormone administration, as shown by Huggins, and a "hormone-poor" environment as a result of the removal of endocrine glands.

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PLATES

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FIGURE 1.—Gross appearance of 2 metastatic tumors developed in the lung in a rat bearing 3-methylcholanthrene-induced mammary cancer.

FIGURE 2.—Microscopic picture of the lung metastases developed in a rat bearing 3-methylcholanthrene-induced mammary cancer. *Note* glandular appearance of the tumor. × 200

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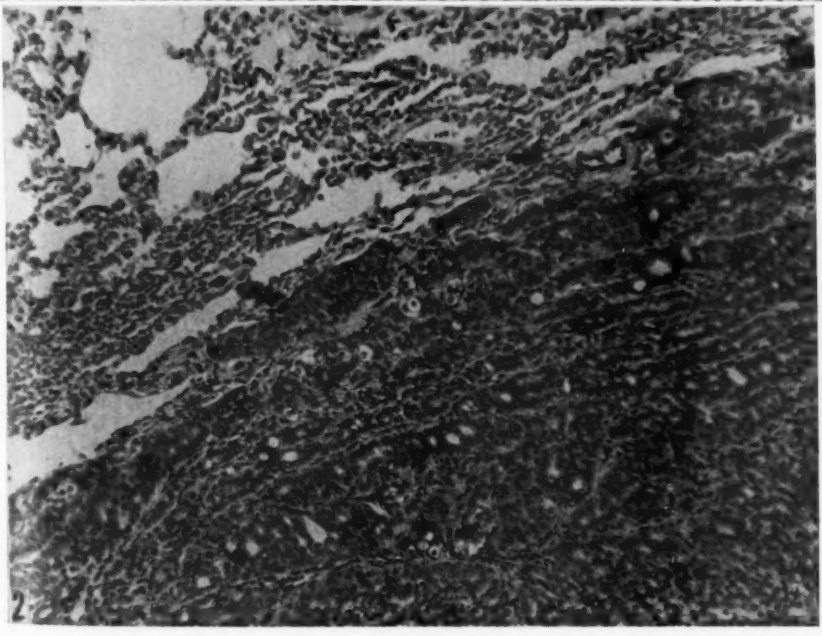

Survival of Patients With Cancer of the Stomach, Connecticut, 1935–54 [1, 2]

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SUMMARY

We are presenting a detailed 10-year survival analysis by stage, age, calendar period of diagnosis, and type of treatment for patients with cancer of the stomach diagnosed in the State of Connecticut between 1935 and 1954. Observed survival is measured relative to expected survival in the general population. Among patients diagnosed 1935–44, about 1 out of 5 who were expected to survive the 1st year after diagnosis, in fact survived; of those expected to live from the end of the 1st year through the 5th year, 1 out of 3 survived. Mortality among the 5-year survivors was far greater than that of persons in the general population of similar age. Patients diagnosed 1945–54 experienced slightly higher 5-year survival than those diagnosed 1935–44; this improvement in length of survival was associated with an increase in the percent of patients treated by surgical intervention and an increase in the survival time of the surgically treated patients. The data suggest that the delay from onset of symptoms to treatment was not shortened, but that mortality from operations was reduced. Five-year survival rates for stomach cancer in Connecticut are similar to the average obtained at 8 treatment centers in the United States.—J. Nat. Cancer Inst. 25: 1005–1021, 1960.

THIS IS the fourth in a series of reports on the survival experience of cancer patients diagnosed in Connecticut hospitals during the 20-year period 1935–54. The first report discussed cancer of the breast and included a detailed exposition of the statistical method used in the analysis (*1*). The second and third discussed cancer of the uterus and ovary (*2*, *3*). The data are based on information submitted by 36 hospitals to the Tumor Registry operated by the Connecticut State Department of Health, the history of which has been described by Griswold and Cutler (*4*) and Greenberg (*5*). An electronic computer available at the National Institutes of Health made this analysis possible.[5]

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[2] This study was made possible, in part, by financial assistance from the National Cancer Institute, National Institutes of Health, Public Health Service. The gathering of the basic data was partially supported by grant 9437; machine processing was supported by contract SA-43-ph-1903.

[3] National Cancer Institute, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[4] Chronic Disease Control Section.

[5] We are indebted to Mr. Elliot Cramer and Mr. Michael C. Altschuler who ably programmed and executed the computations.

The data from the Connecticut Registry are unique in that they provide information on virtually all known cases of cancer in a community, rather than on patients admitted to an individual treatment center. Thus, the data summarized in this report describe the survival experience of the *average* patient with cancer of the stomach. We will compare this experience with results obtained at several medical centers.

MATERIAL

This report contains information on 5,369 cases of cancer of the stomach, 3,438 men and 1,931 women. We excluded patients with a known prior history of cancer, 10 patients of unknown age, all cases first diagnosed at autopsy, and cases for which the death certificate was the sole source of information. Virtually all the patients were white. To provide a basis for evaluating changes over time, the patients were divided into two cohorts according to calendar period of diagnosis: 1935–44 (2,273 patients) and 1945–54 (3,096 patients).

The age distribution of the patients is shown in table 1. Although the men outnumbered the women in the ratio of 1.8 to 1, the two sex groups are strikingly similar in age composition. The marked increase in age from the first calendar period of diagnosis, 1935–44, to the second, 1945–54, is noteworthy: The proportion under age 55 decreased by about 40 percent, and the proportion over 75 nearly doubled. The median age increased from 62 to 66. This shift to older ages at diagnosis parallels that reported by other authors (*6*, *7*) and coincides with the general decline in the incidence of stomach cancer in the United States (*8*).

TABLE 1.—Percentage age distribution by sex and calendar period of diagnosis: patients with cancer of the stomach diagnosed in Connecticut, 1935–54

	Both sexes		Male		Female	
	1935–44	1945–54	1935–44	1945–54	1935–44	1945–54
Number of cases	2, 273	3, 096	1, 473	1, 965	800	1, 131
Percent:						
All ages*	100	100	100	100	100	100
Under 45	8	5	7	5	8	6
45–54	21	12	21	13	20	11
55–64	30	20	32	31	27	25
65–74	32	35	31	35	33	34
75+	10	19	9	17	12	23
Median age	62	66	62	66	63	67
Average age	62	65	61	65	62	66

*Percentages may not total 100 because of rounding.

The sex ratio of 1.8 to 1 is similar to that noted in the 10-city survey (*9*) and to that reported by several large treatment centers (*10–12*), but differs markedly from the 3 to 1 ratio reported by the Mayo Clinic (*13*), the University of Minnesota Hospitals (*6*), and the University of Michigan

Hospital (*14*). The age distribution for the cases at Connecticut is also similar to that observed in the 10-city survey. The patients seen at some treatment centers have been much younger (*7, 10, 13, 15*). These differences in sex and age distribution indicate that the patients seen at some treatment centers are not representative of patients with cancer of the stomach in the United States and suggest that there may be factors of selection. The observed variation in the characteristics of patients in different treatment centers emphasizes the importance of taking patients' characteristics into account in comparing end results. Furthermore, patients in different series may vary with respect to factors of prognostic significance for which no information is available, *e.g.*, socioeconomic status.

Fifty-nine percent of the diagnoses were microscopically confirmed. We included the 2,224 clinically diagnosed cases in this analysis for two reasons: (1) We felt it desirable to evaluate the survival experience of all persons in the Connecticut community that were diagnosed as having cancer of the stomach. (2) The survival rates for the patients whose disease was clinically diagnosed are markedly lower than for those whose disease was microscopically confirmed (*16*). This suggests that the former group is probably made up, in large part, of patients for whom biopsy was considered either medically inadvisable or unnecessary. There is little doubt that a large number of the clinically diagnosed cases were in fact cancer, though the classification by primary site and stage is less certain than in the microscopically confirmed cases. In any event, inclusion of clinically diagnosed cases depresses rather than increases the survival rates. For example, the 1-year survival rate for the total population of males with stomach cancer, diagnosed in 1935–44, was 18 percent, in contrast to a rate of 23 percent for patients with cancer microscopically confirmed. The analysis in this report is based primarily on the total population afflicted with cancer of the stomach, *i.e.*, the confirmed and unconfirmed cases combined; however, because some readers may be interested in the survival rates of the confirmed cases only, we are giving these along with the rates for the total population in tables 5 and 7.

Table 2 summarizes the follow-up information on the 5,369 patients with cancer of the stomach. Only 84 (1.5%) of the patients were lost

TABLE 2.—Follow-up status of patients with cancer of the stomach diagnosed in Connecticut, 1935–54, by 5-year follow-up interval, and calendar period of diagnosis

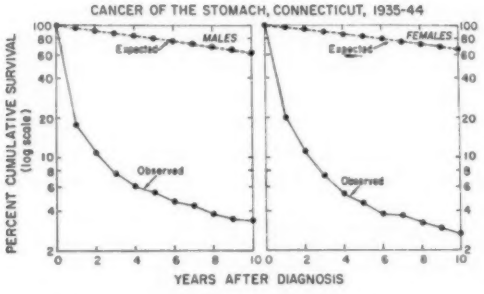
Calendar period of diagnosis and follow-up interval	Alive at beginning of interval	Died during interval	Lost to follow-up during interval	Withdrawn alive during interval*
Patients diagnosed 1935–44				
0–5 years	2, 273	2, 134	30	0
5–10 years	109	40	14	0
Patients diagnosed 1945–54				
0–5 years	3, 096	2, 625	54	322
5–10 years	95	23	3	69

*Alive on closing date of study, December 31, 1954.

during the first 5 years of follow-up. However, of the patients diagnosed in the first period, 1935–44, 14 (13%) of the 109 who were known to have survived the first 5 years were lost between the 5th and the 10th year. Of the patients diagnosed in the second period, 1945–54, only 3 (3%) of the 95 known to be alive at the beginning of the 6th year were lost before the end of the 10th year. Because of the small number of 5-year survivors, the observed survival rates for the 6th through the 10th years are statistically unreliable for patients in both calendar periods.

SURVIVAL EXPERIENCE

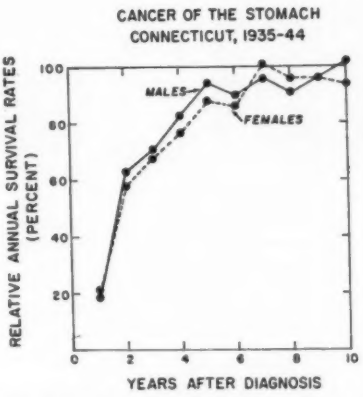
Text-figure 1 shows the survival experience in 10 years of follow-up of the 1,473 male and 800 female patients with stomach cancer diagnosed during 1935–44. The logarithmic scale used for plotting the percentage of survivors at the end of successive follow-up years permits visual evaluation of the rate of depletion (mortality). The very steep slope of the initial part of the "observed" line tells us how badly these patients fared: only 18 percent survived the 1st year; 5.5 percent survived the first 5 years. For comparison, we are showing the expected survival experience for a group of persons from the general population with an age distribution similar to that of the patients with stomach cancer. Visually, the "observed" and "expected" slopes are roughly parallel for men and women after 5 years. This means that the rate of mortality in the patients with stomach cancer declined to the approximate rate in the general population in about 5 years. At that point 95 percent of the patients had died.



TEXT-FIGURE 1.—Observed and expected survival rates for 10 years of follow-up: patients with cancer of the stomach diagnosed in Connecticut, 1935–44.

The *relative annual survival rates* shown in text-figure 2 provide a more precise measure of the relationship between the observed and expected survival rates in successive follow-up periods. The relative survival rate is the ratio of the observed to the expected survival rate. A relative rate of 100 percent means that during a specific follow-up interval the mortality

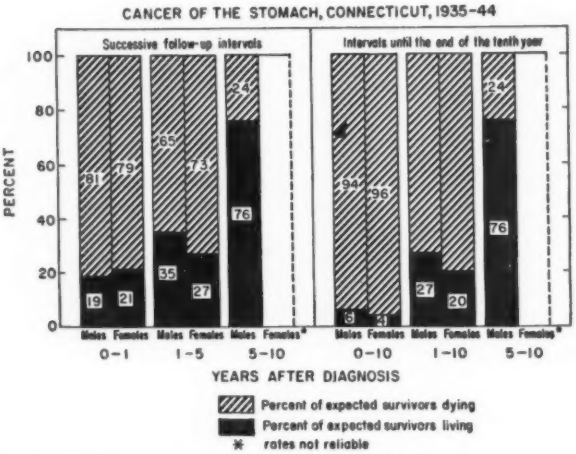
rates in the patient group and in the general population were equal. The attainment and maintenance of annual survival rates in the patient group equal to that in the general population would indicate that excess mortality had been eliminated.



TEXT-FIGURE 2.—Relative annual survival rates for successive follow-up years: patients with cancer of the stomach diagnosed in Connecticut, 1935–44.

From text-figure 2 we see that the annual relative survival rates increase sharply from the 1st-year value of about 20 to about 90 percent for the 5th year. This is in sharp contrast to the rates for cancer of the female reproductive system (*1*, *2*): For cancer of the breast the 1st-year value was 84, for cancer of the cervix 73, and cancer of the corpus 81; the 5th-year values were 93, 96, and 96, respectively. Only 109 of the original 2,273 patients with stomach cancer were left in the study after the first 5 years. The relative rates from the 5th to the 10th year fluctuate around 95 percent. These values, however, should not be taken literally because the annual rates from the 5th to the 10th year are not very reliable statistically due to the small number of cases. Nevertheless, there is sufficient statistical evidence to suggest that excess mortality due to stomach cancer was not eliminated among those fortunate few who survived the first 5 years.

Text-figure 3 summarizes the survival experience of the patients with stomach cancer diagnosed from 1935–44 relative to expectation for successive follow-up intervals. About 1 out of 5 expected survivors survived the 1st year, about 1 out of 3 from the 1st to the 5th year, and about 3 out of 4 from the 5th to the 10th year. The stomach-cancer data parallel our earlier finding for cancer of the female reproductive system that cancer patients who survive 5 years are not necessarily cured (*1–3*): About a quarter of the 5-year survivors who were expected to be alive at the end of 10 years in fact died before that time.



TEXT-FIGURE 3.—Relative survival rates for specified follow-up intervals: patients with cancer of the stomach diagnosed in Connecticut, 1935–44.

Variation by Sex

Table 3 shows that the 1-, 5-, and 10-year survival rates for women with stomach cancer are virtually identical to those of the men, both for the 1935–44 cohort and the 1945–54 cohort.

TABLE 3.—Ten years of survival experience, by sex: patients with cancer of the stomach diagnosed in Connecticut, 1935–44 and 1945–54*
(Rates are expressed as percents)

	Males			Females		
		Relative			Relative	
	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error
1935–44						
0–1	18	19	1.0	20	21	1.5
0–5	6	7	0.8	5	6	0.9
0–10	3	6	0.8	3	4	0.9
1–5	31	35	3.5	23	27	3.6
5–10	*62*	*76*	7.0	n.r.	n.r.	
1945–54						
0–1	25	26	1.0	26	27	1.4
0–5	8	11	1.0	7	9	1.1
0–10	4	7	2.5	4	7	1.7
1–5	33	40	3.5	28	32	3.5
5–10	n.r.	n.r.		n.r.	n.r.	

*Rates in italics have standard errors between 5.0 and 9.9. Rates with standard errors of 10.0 or larger are not shown (n.r. = not reliable).

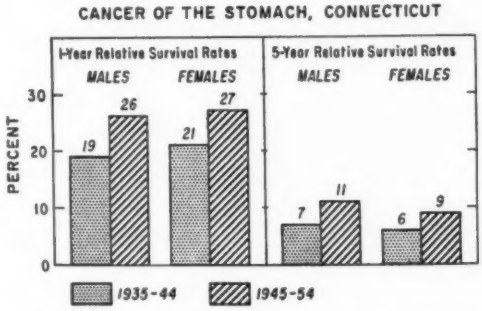
Secular Trend

A number of authors have noted an improvement in rate of survival from stomach cancer in recent decades (*6, 11, 13, 17*). This improvement

was invariably accompanied by a reduction in deaths from operations. It is of interest to compare the results in Connecticut for the two periods of time.

We are evaluating the change in survival over time in Connecticut both in terms of the *crude* and the *relative* survival rate. The relative survival rate corrects for any decrease in deaths from other causes during the second period resulting from the lowering in general mortality in recent years, and also corrects for any increase in deaths from other causes resulting from the increasing age of the population with stomach cancer. Because of the short duration of survival for patients with stomach cancer, normal mortality plays a minor role, and the crude and relative rates for intervals beginning at diagnosis are quite similar.

Although mortality among patients diagnosed in the second period was also exceedingly high, there was some improvement over the first period. For men, the 1-year relative survival rate increased from 19 to 26 percent and the 5-year rate from 7 to 11 percent (table 3 and text-fig. 4). For women, the corresponding increases were from 21 to 27 and from 6 to 9. For both men and women, the improvement appeared to occur at each age (table 8 and text-fig. 5). It is noteworthy that a large part of the improvement in 5-year survival occurred in the 1st year of survival. We will attempt to interpret this improvement in the section on treatment.



TEXT-FIGURE 4.—Relative 1- and 5-year survival rates, by sex: patients with cancer of the stomach diagnosed in Connecticut, 1935–44 and 1945–54.

Variation by Stage of Disease

The classification according to stage of disease is based on all available information at discharge from the hospital. The following definitions were used:

1. Localized—cancer is confined to the site of origin.
2. Regional spread—cancer has passed the bounds of the site of origin; furthest spread thought to be limited to neighboring organs or tissues, or to regional lymph nodes.
3. Distant or remote spread—cancer has spread to organs or tissues beyond those immediately draining or bordering the site of origin.

Table 4 shows the distribution of cases by stage at diagnosis, and table 5 shows 1- and 5-year survival rates for "localized" and "regional" cases.

TABLE 4.—Stage distribution by microscopic confirmation of diagnosis: patients with cancer of the stomach diagnosed in Connecticut, 1935–44 and 1945–54

(Includes 10 patients of unknown age which were excluded from survival computations)

	All stages	Localized	Regional	Distant	Stage unknown
Confirmed and not confirmed, combined 1935–44					
Number of cases	2, 276	859	579	655	183
Percent	100	38	25	29	8
1945–54					
Number of cases	3, 103	735	1, 082	948	338
Percent	100	24	35	31	11
Confirmed only					
1935–44					
Number of cases	1, 064	304	379	344	37
Percent	100	29	36	32	3
1945–54					
Number of cases	2, 085	462	874	668	81
Percent	100	22	42	32	4

TABLE 5.—Crude and relative survival rates by stage of disease and microscopic confirmation of diagnosis: patients with cancer of the stomach diagnosed in Connecticut, 1935–44 and 1945–54

(Rates are expressed as percents)

Sex, confirmation, follow-up interval in years, and period of diagnosis	All stages*		Localized		Regional	
	Crude survival rate	Relative survival rate	Crude survival rate	Relative survival rate	Crude survival rate	Relative survival rate
Males						
Confirmed and not confirmed, combined						
0–1						
1935–44	18	19	27	29	20	21
1945–54	25	26	43	45	29	30
0–5						
1935–44	6	7	11	14	4	5
1945–54	8	11	20	26	7	9
Confirmed only						
0–1						
1935–44	23	24	43	44	25	26
1945–54	30	32	58	61	33	34
0–5						
1935–44	9	11	24	29	5	6
1945–54	11	13	29	36	8	10
Females						
Confirmed and not confirmed, combined						
0–1						
1935–44	20	21	29	30	21	22
1945–54	26	27	42	44	32	33
0–5						
1935–44	5	6	10	13	2	2
1945–54	7	9	17	21	7	9
Confirmed only						
0–1						
1935–44	29	29	50	51	23	23
1945–54	31	32	59	61	37	38
0–5						
1935–44	8	9	20	23	3	4
1945–54	10	12	25	31	9	11

*Includes "distant" and "stage unspecified."

Stage of disease was, of course, an important factor in prognosis. The *localized* cases had a decidedly better outlook than the *regional* cases, and the latter, as can be seen from a comparison with *all stages*, had a decidedly better outlook than the *distant* cases. Both for patients with localized and regional spread of disease, rate of survival improved from the first to the second period. The very marked excess in survival rate of the "localized" over the "regional" cases parallels the findings for cancer of the female reproductive system.

Classification by stage of disease is quite uncertain for the unconfirmed cases; therefore we show the data by stage of disease for the "confirmed and not confirmed, combined" and for the "confirmed only." It is likely that many cases staged clinically as "localized" were in fact metastasized. We note that the difference in survival between "localized" and "regional" cases is much greater for "confirmed only" cases than for "confirmed and not confirmed, combined." This is explained by the highly selective nature of the confirmed cases: For example, among the "localized" cases only 2 out of 10 were untreated, as against 9 out of 10 for the unconfirmed cases.

Delay Before Treatment

It is generally believed that the delay before treatment is a crucial factor in the management of stomach cancer. Stomach cancer is often a rapidly progressing disease; therefore early diagnosis and treatment are of the utmost importance. Welch and Allen (*18*) expressed the belief that if all patients were subjected to resection as soon as carcinoma of the stomach developed, all would be cured. A number of factors are responsible for the delay before treatment. First is the insidious nature of the disease: There are no symptoms in the early stages (*7*, *19*). Second is neglect of symptoms. Third is the difficulty in differentiating stomach cancer from benign gastric ulcer (*18*, *20*).

Although there is no supporting evidence, a number of authors have claimed that the cancer-education campaigns of recent years have increased the alertness of patients and physicians to symptoms of cancer, shortening the delay between onset of symptoms and diagnosis. Welch and Wilkins (*17*) reported a decrease in median duration from onset of symptoms of cancer of the stomach to treatment at the Massachusetts General Hospital from 5 months in 1937–46 to 4 months in 1947–56. Shahon, Horowitz, and Kelly (*6*) found no significant shortening in delay from onset of symptoms of stomach cancer to surgical treatment at the University of Minnesota Hospitals from 1936 to 1949. The Connecticut data contain no information on the interval from onset of symptoms to treatment, but earlier case findings, if any, would be reflected in the percentage of cases classified as localized at diagnosis. Surprisingly, this percentage decreased sharply from 38 in the first period to 24 in the second. Among patients whose diagnoses were microscopically confirmed, the percent localized decreased from 29 to 22 percent. These decreases

occurred at each age group where the number of cases was sufficient for evaluation. We doubt that this is a bona fide shift toward later diagnosis. The percentage of patients treated surgically increased from the first to the second period, allowing greater opportunity to discover metastases. If there has been a trend in Connecticut, as elsewhere (*6*, *17*), toward more radical operations involving adjacent organs or lymph nodes, this would allow still greater opportunity to discover metastases.

Treatment

The patients were classified into three broad categories according to the treatment they received during the first course of medical care: surgery, other treatment, and no treatment. The method of therapy for virtually all patients with stomach cancer that were treated was surgery. Other methods of treatment were used in only about 2 percent of the cases (table 6). Treatment, as defined by the Connecticut Tumor Registry, consists of procedures directed toward the tumor, whether for cure or palliation. Thus, an exploratory laparotomy would *not* be considered treatment, whereas a palliative subtotal gastrectomy *would*. The data at hand contain no information on the extent (radical or subtotal) or the intent (cure or palliation) of surgery.

TABLE 6.—Percentage distribution according to treatment during first course of therapy, by sex and period of diagnosis: patients with cancer of the stomach diagnosed in Connecticut, 1935–54

	Total number of cases	Percent			
		Total treated and not treated	Surgery	Other treatment	Not treated
Confirmed and not confirmed, combined					
Males					
1935–44	1,473	100	35	3	62
1945–54	1,965	100	46	2	52
Females					
1935–44	800	100	32	2	66
1945–54	1,131	100	46	2	52
Confirmed only					
Males					
1935–44	728	100	55	4	41
1945–54	1,343	100	61	2	37
Females					
1935–44	336	100	58	5	37
1945–54	738	100	63	2	35

Table 6 shows that the percent of patients treated surgically increased markedly from about 35 in 1935–44 to 46 in 1945–54; even so, in the later period more than half the patients were untreated. In view of the fact that the 5-year survival rate for untreated cancer of the stomach is virtually nil (table 7), the increase in the number of treated patients should

alone account for an improvement in the over-all survival rate, unless the increased use of surgical intervention was accompanied by riskier procedures. An inspection of table 7 indicates that, on the average, procedures in the second period were not riskier, but safer. The 1-year relative-survival rate for the resected patients increased from 34 to 44 in men and from 38 to 45 in women; the 5-year rates increased correspondingly. Thus, at least two factors are responsible for the improvement in rate of survival from the first to the second period: (1) greater use of surgery, and (2) improvement in rate of survival of the surgically treated patients.

TABLE 7.—Relative survival rates by treatment during first course of medical care, sex, and period of diagnosis: patients with cancer of the stomach diagnosed in Connecticut, 1935–54*

(Rates are expressed as percents)

	Total, treated and not treated		Surgery		Not treated	
	Relative survival rate	Standard error	Relative survival rate	Standard error	Relative survival rate	Standard error
Confirmed and not confirmed, combined						
Males						
1935–44						
0–1 year	19	1.0	34	2.2	10	1.0
1–5 years	35	3.5	47	4.3	*15**	7.3
0–5 years	7	0.8	16	1.8	1	0.5
1945–54						
0–1 year	26	1.0	44	1.7	11	1.0
1–5 years	42	3.5	43	3.7	*23*	7.8
0–5 years	11	1.0	19	1.8	2	0.7
Females						
1935–44						
0–1 year	21	1.5	38	3.1	11	1.4
1–5 years	27	3.6	*32*	5.3	*21*	6.8
0–5 years	6	0.9	12	2.2	2	0.7
1945–54						
0–1 year	27	1.4	45	2.3	12	1.4
1–5 years	32	3.5	38	4.5	*16*	7.0
0–5 years	9	1.1	17	2.2	2	0.9
Confirmed only						
Males						
1935–44						
0–1 year	24	1.6	38	2.5	4	1.2
1–5 years	46	4.5	47	4.5	*19*	9.9
0–5 years	11	1.3	18	2.1	1	0.6
1945–54						
0–1 year	32	1.3	46	1.8	8	1.3
1–5 years	41	3.7	46	4.0	*10*	6.8
0–5 years	13	1.3	21	2.0	1	0.7
Females						
1935–44						
0–1 year	29	2.5	41	3.6	6	2.1
1–5 years	*30*	5.0	*34*	5.8	0	—
0–5 years	9	1.7	14	2.7	0	—
1945–54						
0–1 year	32	1.8	47	2.4	7	1.6
1–5 years	38	4.6	38	4.7	0	—
0–5 years	12	1.6	18	2.4	0	—

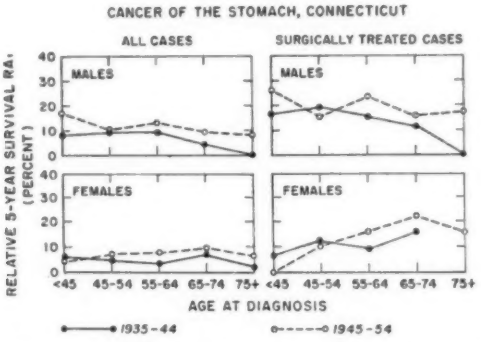
*Rates in italics have standard errors between 5.0 and 9.9.

The latter is probably a combination of such factors as improved surgical techniques and better control of infection. The rates for the 1- to 5-year interval for the surgically treated patients remained stable. The improvement in the 5-year survival rate resulted directly from the improvement in the 1-year rate, which suggests that reduction of deaths from operations may have been an important factor.

We have suggested that infections were controlled more effectively in the second period. The 1945–54 period covers the postwar era when the use of antibiotics became widespread. Also, there is little doubt that the quality of surgery was better in the later period, *i.e.*, that there were more and better-trained surgeons: the 1940 Directory of Medical Specialists lists 36 Connecticut physicians certified by the American Board of Surgery, and the 1951 Directory lists 101 such physicians. However, nothing in the Connecticut data suggests that the delay from onset of symptoms to treatment was reduced. One might reasonably anticipate that a significant shortening in delay of treatment would effectively reduce the mortality between the 1st and the 5th years of follow-up. This did not occur. The fact that the percent of cases classified as "localized" at diagnosis decreased from the first to the second period casts further doubt on the hypothesis that delay has been reduced.

Age

To evaluate the relationship between survival and age, we took normal survival expectancy into account. Text-figure 5 and table 8 show relative 5-year survival rates by age at diagnosis for each of 5 age groups; table 8 also shows the corresponding crude rates. Some of the age-specific rates, as indicated by the standard errors, are based on a relatively small number of cases and therefore are not reliable. In general, the age-variation in survival does not appear to be very marked, though there is a mild suggestion that mortality from cancer of the stomach increases



TEXT-FIGURE 5.—Five-year relative survival rates by age at diagnosis: patients with cancer of the stomach diagnosed in Connecticut, 1935–44 and 1945–54. (The rate for the female cases treated surgically, age 75+, diagnosed 1935–44, is statistically unreliable and therefore not shown.)

TABLE 8.—Five-year survival rates by age, first course of therapy, calendar period of diagnosis, and sex: patients with cancer of the stomach diagnosed in Connecticut, 1935–54

(Rates are expressed as percents)*

Sex, therapy, and period of diagnosis	Under 45			45–54			55–64			65–74			75 and over		
		Relative			Relative			Relative			Relative			Relative	
	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error
Males															
Treated and not treated, combined															
1935–44	8	8	2.8	8	9	1.7	7	9	1.5	3	4	1.1	0	0	—
1945–54	16	17	4.8	10	10	2.4	11	13	1.8	6	9	1.5	4	8	2.5
Surgery															
1935–44	*16*	*17*	6.2	18	19	3.5	14	16	3.0	9	12	3.6	0	0	—
1945–54	*26*	*27*	6.9	15	16	3.7	21	24	3.3	12	16	3.2	*10*	*18*	6.5
Females															
Treated and not treated, combined															
1935–44	6	6	3.0	5	5	1.8	3	4	1.4	6	8	1.9	1	3	2.5
1945–54	5	5	3.4	6	7	2.5	8	8	2.0	10	12	2.2	4	7	2.9
Surgery															
1935–44	7	7	4.6	12	13	4.3	8	10	3.4	*13*	*17*	5.7	n.r.	n.r.	—
1945–54	0	0	—	10	11	4.5	15	17	3.7	19	23	4.3	*10*	*17*	7.1

*Rates in italics have standard errors between 5.0 and 9.9. Rates with standard errors of 10.0 or larger are not shown (n.r. = not reliable).

with age in men and decreases with age in women. Prognosis in stomach cancer is not good at any age.

Comparison With Other Series

Comparison of results reported by different investigators is very difficult, because patients in various series are often not comparable. Superior results achieved in one treatment center as contrasted with another may be due to differences between the two groups of patients in age, race, socioeconomic status, general clinical condition, and the extent of the cancer at diagnosis. Insufficient detail is usually reported to permit adjustment for differences with respect to all these factors. The results are frequently not reported uniformly and there may be differences in the definition of terms. In addition, one series may include all diagnosed cases, treated and untreated, and another series may be restricted to cases treated in a specified manner. Perhaps the chief difficulty in comparing various series is that the reporting of end results is quite variable with respect to inclusion or exclusion of outpatients, deaths from operations, patients who refused treatment, etc. The Joint Committee on Reporting Cancer End Results (*21*) has recommended minimum rules which include complete accounting of all patients seen, both treated and untreated. We hope that more investigators will apply these principles in the future.

Despite these limitations, comparisons are interesting and therefore frequently attempted. Shahon, Horowitz, and Kelly (*6*) have compiled the results for various centers, and we have reproduced part of their compilation in table 9, excluding data for patients seen before 1930 and adding more recent data from the Massachusetts General Hospital (*17*). We are showing for comparison the Connecticut experience, which offers a base of background information enabling an evaluation of hospital data. We are omitting from the table of Shahon *et al.* the column showing percent resection survivors because this information is not comparable with the Connecticut data. In computing the percent resection survivors, many of the hospitals shown in table 9 excluded the hospital deaths. The Connecticut survival data include the hospital deaths.

Table 9 shows that the increases observed in Connecticut in percent treated surgically and in survival have taken place elsewhere. It is noteworthy that the percent of the patients with stomach cancer resected ranged from as little as 7.7 percent (Los Angeles Hospital, 1930–39) to as much as 66.7 percent (Massachusetts General Hospital, 1947–53). The percent resected at Connecticut was in line with, though somewhat higher than, the average for the 8 centers. The wide variation in percent resected clearly reflects the heterogeneity of patient populations seen at different hospitals and differences in the definition of resection.

The last column of table 9 shows that the over-all 5-year survival rate for Connecticut was somewhat less than the average for the 8 centers (6.7 vs. 8.2). The Mayo Clinic, Rochester, Minnesota, Mt. Sinai Hos-

TABLE 9.—Comparison of survival from cancer of the stomach in Connecticut, 1935–54, with that in various medical centers, 1930–53*

Institution	Calendar period	Number of cases	Percent resected	Crude 5-year survival rate (*percent*)
Connecticut, 36 hospitals, average†	1935–54	5, 369	40. 9	6. 7
Males	1935–44	1, 473	35	5. 5
	1945–54	1, 965	46	8. 2
Females	1935–44	800	32	4. 6
	1945–54	1, 131	46	7. 2
Various medical centers, average†	1930–53	11, 274	34. 0	8. 2
Mayo Clinic	1940–49	4, 000‡	44. 0	14. 0
Cleveland Clinic	1940–45	406	25. 0	7. 1
Los Angeles Hospital	1930–39	1, 295	7. 7	1. 4
	1940–49	1, 596	12. 8	
Mt. Sinai Hospital, N.Y.	1938–42	157	41. 0	6. 4
	1943–47	187	52. 0	11. 6
University of Illinois	1931–47	1, 004	31. 9	4. 7
Ochsner Clinic	1942–53	153§	37. 0	7. 2
University of Michigan	1934–40	609	35. 9	8. 2
	1941–46	655	36. 3	7. 0
Mass. General Hospital	1937–41	375	46. 0	n.a.‖
	1942–46	457	53. 0	7. 0
	1947–52	380	66. 6	12. 9

*Source: The data for the various medical centers are from a table given by Shahon, Horowitz, and Kelly (*6*), except for Massachusetts General Hospital, 1947–52, the data for which are from Welch and Wilkins (*17*). Excluded from the table by Shahon, Horowitz, and Kelly are data for patients treated before 1930.

†The percentages were weighted in proportion to the number of cases.

‡Number of cases estimated from data given by Berkson *et al.* (*15*).

§Number of cases estimated from data given by Ochsner *et al.* (*10*).

‖Abbreviation: n.a. = not available.

pital, New York, New York, and Massachusetts General Hospital, Boston, Massachusetts, show the highest over-all rates: between 11 and 14 percent of the patients with stomach cancer survived 5 years. If the Mayo Clinic experience is excluded, the average 5-year survival rate for the remaining 7 centers is only 4.9 percent, compared with 6.7 percent for Connecticut.

DISCUSSION

Despite declining incidence, cancer of the stomach continues to be a major killer. In 1958, 21,033 persons succumbed to the disease in the United States, which accounted for about 1 in every 4 deaths from cancer (*22*). And despite some improvement in length of survival, the over-all chances of surviving stomach cancer for 5 years are at most 10 or 15 in 100.

Surgery is the only presently available method of treatment that offers hope of cure in cancer of the stomach. Ochsner's report illustrates the difficulties confronting the surgeon (*10*): "In the present series of 247 patients, 33 were inoperable when first seen. . . . There were 214 patients whom we considered operable, of whom 16 refused surgery. Of 198 who were explored, 107 were found to be nonresectable. . . . Of 91 patients in whom a resection was done, in 60 the lesion was advanced

and a palliative procedure was done. . . . There were only 31 in whom, presumably, a curative resection was done. Of the 31 patients . . . 2 died (in the hospital)." Effectively, only 29 out of 247 patients (12%) had a chance for cure.

In spite of the increase in the percent of patients treated surgically, from 35 to 46, and an increase in 5-year survival for these patients, the over-all rates in Connecticut changed by only a few percentage points. It is clear that present methods of treating stomach cancer are inadequate, partly because more than half the patients are not treated and a large number of the remaining patients are treated with palliative intent only, and partly because the results for the treated patients are so poor. Ochsner and Abbott (*20*) have urged a concerted effort against "one of the prime scourges of mankind": (1) earlier diagnosis, (2) excision of all gastric ulcers, whether benign or malignant, (3) resection of other apparently benign and premalignant conditions, *e.g.*, gastric polyps and certain cases of persistent chronic gastritis, (4) better techniques for visualization and biopsy, and (5) experimental and statistical investigations of possible etiologic factors. Further research in methods of treatment, particularly in adjuvant therapy, should not be overlooked in any concerted effort against cancer of the stomach.

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Mammary Tumors and Mammary-Gland Development in Hybrid Mice Treated With Diethylstilbestrol for Varying Periods [1, 2]

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SUMMARY

Male and female mice, F_1 hybrids of strains C3H and RIII, were implanted with diethylstilbestrol pellets for varying periods. The mammary-tumor incidence was high in untreated as well as in estrogen-treated virgin females of reciprocal crosses. Normal C3H × RIII females developed mammary tumors at an early age; tumors appeared sooner when the mice were exposed to a pellet of diethylstilbestrol for 8 weeks, but prolonged treatment did not further reduce the mean tumor age. Normal RIII × C3H females developed tumors much later than the reciprocal cross; the mean tumor age was greatly reduced by exposure to a pellet for 8 weeks and still further reduced by treatment for life. C3H × RIII males exposed to a pellet for 6 or 8 weeks had a much higher incidence of tumors and a lower mean tumor age than RIII × C3H males with similar treatment. With prolonged diethylstilbestrol treatment both hybrids had a high incidence of tumors; the tumors developed slightly earlier in C3H × RIII males. The differences in incidence of mammary tumors and in mean tumor age between the two hybrid groups suggest that the agent in the milk of strain C3H is more active than that of RIII. Whole mounts of mammary glands of mice treated with diethylstilbestrol showed dilated ducts, clusters of distended acini, and a characteristic lack of uniformity in different areas. Within 4 to 6 weeks after removal of the pellets, the glands of females resembled those of normal mice; the ducts of male glands became narrow and the acini regressed almost completely except for those organized into nodules.—J. Nat. Cancer Inst. 25: 1023–1039, 1960.

MANY INVESTIGATORS have induced mammary tumors in male mice, or increased the incidence in nonbreeding females, by means of estrogen stimulation (*1*). However, few workers have attempted to find the effects of treating mice for short periods. Burns and Schenken (*2*) injected estradiol benzoate in C3H male mice weekly and found that a small percentage of the mice developed tumors after treatment for 8 weeks, that a relatively high percentage of tumors occurred after treatment for 16 weeks,

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[2] This investigation was supported in part by research grant C-2661 from the National Cancer Institute, National Institutes of Health, Public Health Service, a grant by the Elsa U. Pardee Foundation, and a gift from Miss Moyah Clark.

but that the incidence was not further increased by 20 or more weekly injections.

Shimkin and White (*3*) found that implants of solid diethylstilbestrol-cholesterol pellets containing 25 percent of the hormone remained in the subcutaneous tissue of mice for over a year without apparent reduction in size. The analysis for diethylstilbestrol in the pellets that were removed after 12 months showed that diethylstilbestrol was not selectively absorbed from the diethylstilbestrol-cholesterol pellets. Richardson (*4*) published the results obtained by implanting diethylstilbestrol-cholesterol pellets into male and female F_1 hybrids of RIII $\times$ C57L mice and removing the pellets after 4, 6, or 8 weeks or leaving them in for the life of the animal. The virgin females of this cross had a fairly high incidence of mammary tumors (63%), but they appeared late in life. The mean tumor age was 21.3 months. The incidence of tumors in females was increased by treatment for 4 or 8 weeks; the mean tumor age became progessively less as the period of treatment increased. Only 10 percent of the males developed mammary tumors after treatment for 6 or 8 weeks; 84 percent developed tumors when treated for life. In a paper published recently by Richardson and Hummel (*5*) on the occurrence of mammary tumors in virgin female mice of strains C3H, RIII, and their F_1 hybrids, it was stated that the mean tumor age in C3H $\times$ RIII females was less than in either parental strain but more closely resembled that of C3H, and the mean tumor age in RIII $\times$ C3H females was greater than in strain C3H but not significantly different from that in RIII mice.

The experiments of Silberberg and Silberberg (*6*) indicated that there is a decrease with advancing age in the susceptibility of the mammary tissue of male mice to estrogenic hormones. They injected estradiol benzoate, weekly for 5 months, into strain C3H males. Tumors occurred in 15.8 percent of the mice when treatment was begun at 4 weeks of age, but only 4.0 percent of the mice developed tumors when treatment was started at 4 months of age. These results are not in accord with those we obtained by implanting diethylstilbestrol pellets into normal male mice at 2 and 6 months of age.

Shimkin (*1*) stated that tumors often appeared at an earlier date in estrogen-treated females than in breeding females of the same strains. We have found a tumor incidence of 95 percent in C3H $\times$ RIII force-bred females (56 mice) at a mean tumor age of 6.8 months.

In this experiment, male and female mice of reciprocal crosses between strains C3H and RIII were exposed to diethylstilbestrol pellets for varying periods; it will be shown that there is a difference in the way these hybrid groups responded to the treatment. These results also differ from those obtained previously with F_1 hybrids of RIII $\times$ C57L mice implanted with diethylstilbestrol pellets.

MATERIALS AND METHODS

Most of the mice used in this study were F_1 hybrids of strains C3H/HeJ and RIII/J. A small number of F_1 hybrids of RIII/J $\times$ C57L/J was also

used. Strains C3H and RIII harbor the milk agent and have high incidences of mammary tumors in breeding females.

Solid pellets of diethylstilbestrol-cholesterol were prepared by the method described by Shimkin and White (*3*) and were implanted subcutaneously by means of a 12-gauge trocar. Each pellet weighed approximately 8 mg. and contained 20 percent diethylstilbestrol and 80 percent cholesterol. Pellets were implanted into male and female mice at 2 months of age, unless otherwise stated, and were removed after 4, 6, or 8 weeks, or were left in for the remainder of the animal's life. When mammary tumors were visible to the naked eye, the animals were killed by chloroform and a section of each tumor was taken for histologic diagnosis. Animals without visible mammary tumors were killed and necropsied when death from other causes seemed imminent.

To compare the structure of the mammary glands of the various groups, the skins with adherent glands were removed from untreated females without tumors; from mice killed after treatment with diethylstilbestrol for 4, 6, or 8 weeks; from mice which were exposed to a pellet for 4, 6, or 8 weeks and later developed tumors; and from tumor-bearing mice exposed to a pellet for life. All glands were equally stimulated by diethylstilbestrol, regardless of the location of the implanted pellet, but third glands were selected for making whole mounts.

OBSERVATIONS

Mammary Tumors

All tumors were diagnosed histologically as adenocarcinomas. Table 1 summarizes the data on incidence of tumors and mean ages when they occurred in F_1 hybrids of reciprocal crosses between strains C3H and RIII when treated with diethylstilbestrol for varying periods. The table also includes the data published in an earlier paper (*5*) on the occurrence of tumors in untreated virgin females of both crosses. The percentage of tumors was very high in untreated as well as in estrogen-treated females of both crosses. Since these hybrids tolerate diethylstilbestrol well, few deaths were attributed to the effects of the treatment. Of greater interest is the increase in rate of tumor development in females exposed to pellets for varying periods. The mean tumor age of untreated C3H $\times$ RIII females (8.5 months) was only slightly greater than in females treated for 4 weeks (7.8 months); this difference is not significant but close to the 0.05 level. The mean tumor age of females treated for 8 weeks (5.8 months) was almost identical to that of females treated for life (6.0 months). In the reciprocal cross, RIII $\times$ C3H, the mean tumor age of untreated females (17.6 months) was much greater than in females treated for 8 weeks (9.3 months). The mean tumor age was still lower in females treated for life (7.6 months).

TABLE 1.—Occurrence of mammary tumors in F_1 hybrids of C3H and RIII mice exposed to diethylstilbestrol pellets for varying periods

Cross ♀ × ♂	Sex	Time exposed to pellets (*weeks*)	Number of mice	Animals with mammary-gland tumors				
				Percent	χ^2 Test		Mean age (*months*)	*t* Test
					χ^2	*P*		*t* *P*
C3H × RIII*	♀	0	101	97	0.03	>0.05	8.5	1.80 >0.05
C3H × RIII	♀	4	61	98			7.8	
C3H × RIII	♀	6	0	—			—	4.26 <0.01†
C3H × RIII	♀	8	58	98	2.01	>0.05	5.8	
C3H × RIII	♀	For life	56	93			6.0	
RIII × C3H*	♀	0	62	84			17.6	
RIII × C3H	♀	4	0	—	2.58	>0.05	—	
RIII × C3H	♀	6	0	—			—	
RIII × C3H	♀	8	49	94	0.14	>0.05	9.3	2.93 <0.01†
RIII × C3H	♀	For life	44	95			7.6	
C3H × RIII	♂	4	45	7			11.8	
C3H × RIII	♂	6	66	61	4.04	<0.05†	9.5	1.94 =0.05
C3H × RIII	♂	8	50	78			7.9	
C3H × RIII	♂	For life	73	97	11.57	<0.01†	6.5	3.04 <0.01†
RIII × C3H	♂	4	0	—			—	
RIII × C3H	♂	6	47	19			17.3	1.28 >0.05
RIII × C3H	♂	8	61	34			14.3	
RIII × C3H	♂	For life	62	94			8.3	

*From data in published report (*5*).
†Indicates the difference is significant.

When the two reciprocal crosses were compared, the mean tumor age of RIII × C3H females treated for life was almost as short as that of C3H × RIII females with similar treatment; the difference, however, is significant ($t = 4.32$; $P<0.01$).

The incidence of tumors in the F_1 hybrid groups of C3H × RIII males increased progressively as the period of treatment increased. The low incidence in males implanted with pellets for only 4 weeks (7%) shows this is close to the shortest period of treatment which will induce the formation of any tumors. There was a sharp increase in the tumor incidence of males treated for 6 weeks (61%). The incidence was higher after treatment for 8 weeks (78%) and still higher after treatment for life (97%). The differences between the 4 groups are significant.

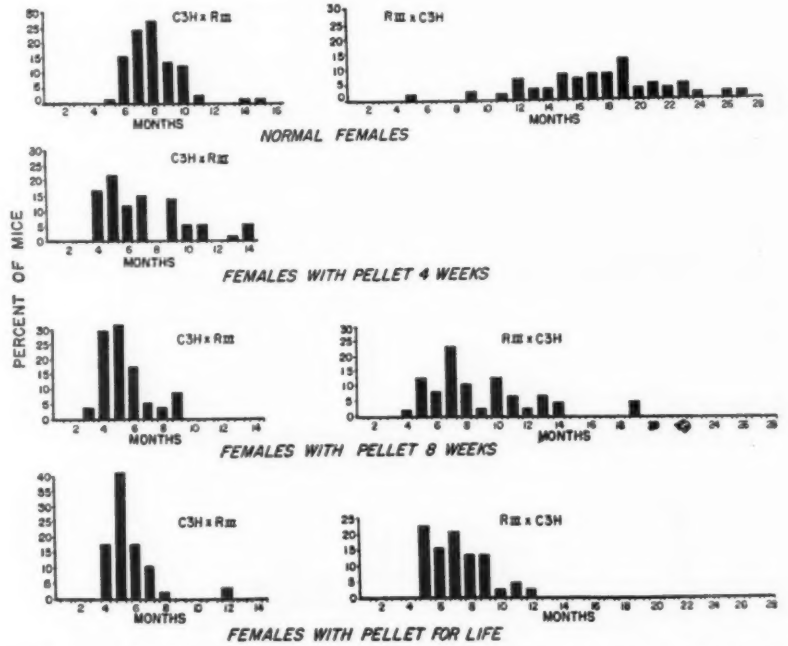
The mean tumor ages of the C3H × RIII males decreased progressively as the period of treatment increased. The mean tumor age of the few males that had tumors after 4 weeks of treatment was 11.8 months. The difference in the mean tumor age of mice treated for 6 weeks (9.5 months) and that of mice treated for 8 weeks (7.9 months) is of doubtful significance ($t = 1.94$; this is very close to the 0.05 level). The mean tumor age of mice treated for 8 weeks was significantly greater than in mice treated for life (6.5 months).

The incidence of tumors in RIII × C3H males treated for 6 weeks

(19%) or for 8 weeks (34%) was much lower than in the males of the reciprocal cross. However, when they received continuous estrogen stimulation, the percentage of tumors (94%) was nearly as high as in C3H × RIII males with a pellet for life (97%). The difference is not significant ($\chi^2 = 1.12$; $P > 0.05$).

The mean tumor age of the RIII × C3H males treated for 6 weeks (17.3 months) was not significantly greater than in males treated for 8 weeks (14.3 months), but the mean tumor age was much less in males receiving continuous estrogen stimulation (8.3 months).

The histogram (text-fig. 1) compares the proportion of tumors found at monthly intervals in virgin females of the two hybrid groups. It was previously stated that in C3H × RIII hybrids the difference between the mean tumor age of untreated females (8.5 months) and that of females treated for 4 weeks (7.8 months) was not significant but close to the 0.05 level. The histogram shows that only 1 percent of the untreated females had tumors before they were 6 months old compared to 38 percent of the females exposed to a pellet for 4 weeks, which indicates that this period of treatment caused acceleration of tumor formation in some of the mice. In the females treated for 6 weeks, or for life, approximately 60 percent of the mice developed tumors before they were 6 months old.

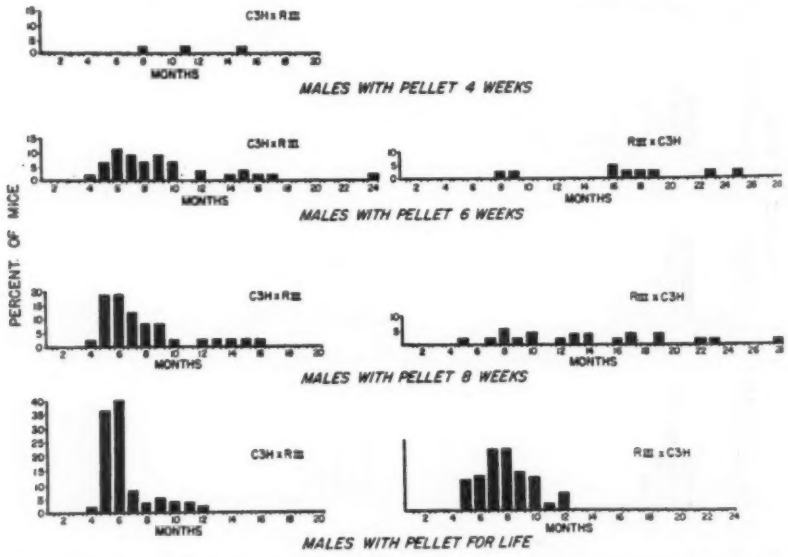


TEXT-FIGURE 1.—Histogram showing percentage of mammary tumors at the age in months when they occurred in normal and diethylstilbestrol-treated females of F_1 hybrids between C3H and RIII.

In the reciprocal cross the RIII × C3H females receiving no diethylstilbestrol developed tumors much later than in the group treated for 8 weeks. Treatment for life further reduced the age when tumors occurred. A comparison of the two hybrid groups shows there is a great difference in the tumor ages of the untreated females but only a small difference in females treated with diethylstilbestrol for life.

The histogram (text-fig. 2) compares the proportion of tumors at monthly intervals in diethylstilbestrol-treated males of the two hybrid groups. A very small number of the C3H × RIII males treated for 4 weeks had tumors, but 60 percent of those treated for 6 weeks developed tumors. The percentage of animals developing tumors before they were 7 months old was as follows: 0 with treatment for 4 weeks, 18 percent with treatment for 6 weeks, 38 percent with treatment for 8 weeks, and 77 percent with treatment for life. A comparison of the two hybrid groups shows that a much smaller percentage of tumors occurred in RIII × C3H males after treatment for 6 or 8 weeks, and they occurred later than in the reciprocal cross. In males treated for life, the percentage of animals with tumors was not significantly different in the two crosses ($\chi^2=1.11$; $P> 0.05$) but the mean tumor age was slightly greater in the RIII × C3H males.

Work is in progress to find the effect of exposing RIII × C3H mice to pellets for 4 weeks. It is possible that the rate of tumor formation in females will be unquestionably accelerated by this treatment since exposure to pellets for 8 weeks greatly decreased the mean tumor age.



TEXT-FIGURE 2.—Histogram showing percentage of mammary tumors at the age in months when they occurred in diethylstilbestrol-treated males of F_1 hybrids between C3H and RIII.

Pellets were implanted into mice 2 months old in most of these experiments. At this age they tolerated the treatment better than younger mice. To find out whether or not the mammary tissue of older mice would be as susceptible to estrogen treatment, the pellets were implanted into male mice 6 months old. Table 2 gives the results of this experiment. As previously stated, a high percentage (97%) of C3H × RIII males developed tumors when the treatment was started at 2 months of age and the pellets were left in for the life of the animal. The mean time of exposure to the pellets was 4.5 months. When treatment was started at 6 months of age, the percentage of mice with tumors (91%) was very high and the mean time of exposure to the pellets (5.4 months) was only slightly greater than in mice receiving treatment at 2 months of age.

TABLE 2.—Occurrence of mammary tumors in male mice implanted with diethylstilbestrol pellets at different ages and treated for life

Cross ♀ × ♂	Age at start of treatment (*months*)	Number of mice	Percent with tumors	Mean tumor age (*months*)	Mean time exposed to pellet (*months*)
C3H × RIII	2	73	97	6.5	4.5
C3H × RIII	6	55	91	11.4	5.4
RIII × C57L*	2	230	84	10.9	8.9
RIII × C57L	6	31	77	13.4	7.4

*From data in published report (*4*).

In an earlier study (*4*) RIII × C57L males were implanted with diethylstilbestrol pellets at 2 months of age. The results, included in table 2, show that a high percentage of mice (84%) developed tumors; the tumors were visible at a mean time of 8.9 months after implantation of the pellet. In the present study the pellets were implanted at 6 months of age and the percentage of tumors (77%) was somewhat lower; the tumors were visible at a mean time of 7.4 months after implantation of the pellets. This was slightly less than in males receiving the pellets at 2 months of age. It may be concluded that in these two groups the mammary tissue of both young and old male mice was very susceptible to treatment with 20 percent diethylstilbestrol-cholesterol pellets and that age was not an important factor in the induction of tumors with this estrogen.

Mammary-Gland Development

Whole mounts were made of third glands of 650 mice. Table 3 compares the percentage of glands of female mice in visually graded groups ranging from 0, which indicates very few lateral buds, to 4, which indicates profuse acinar development. Since the number of glands observed in some of the series was rather small, no attempt was made to analyze the data statistically to determine whether or not there were significant differences between the various groups. A few general conclusions may be drawn from the limited amount of data presented in the table. Many of the glands in C3H × RIII females, 3 months old, showed little acinar

development; in nearly half of the animals the glands were composed mainly of ducts with very few lateral buds. At 4 months of age the glands of most of the females were as well developed as those in females 6 months old. The acinar development in the glands of the 6-month-old females, which were tumor-free, was similar to that of tumor-bearing females 5 to 12 months old. The glands of females that were exposed to diethylstilbestrol for 8 weeks and later developed tumors were similar to those of untreated females that developed tumors. In the reciprocal cross, RIII × C3H, the acinar development in the glands of untreated females was similar to that of females that developed tumors after exposure to a pellet for 8 weeks. Richardson and Hummel (*5*) observed that the mammary glands of normal C3H × RIII virgin females were similar to those of RIII × C3H virgins in the extent of lateral budding and acinar development.

TABLE 3.—Percentage of mammary glands of female mice in visually graded groups ranging from 0, which indicates very few lateral buds, to 4, which indicates profuse acinar development

Cross ♀ × ♂	Time exposed to pellet (*weeks*)	Number of glands observed	Number of mice with tumors	Age range (*months*)	Percentage of glands in grade:			
					0	1 & 2	3	4
C3H × RIII	0	36	0	3–3	45	47	8	
C3H × RIII	0	37	0	4–4	0	65	22	13
C3H × RIII	0	27	0	6–6	0	55	30	15
C3H × RIII*	0	61	61	5–12	0	52	28	20
C3H × RIII	8	49	49	4–9	0	51	33	16
RIII × C3H*	0	21	8	6–12	0	48	38	14
RIII × C3H	8	31	31	5–12	0	49	32	19

*Data taken from published report (*5*).

The photomicrographs illustrate the architecture of the mammary glands in normal and treated mice. Figure 1, the gland of a normal female 3 months old, shows few lateral buds. Figure 2, the gland of a female 3 months old that was killed after exposure to a pellet for 4 weeks, shows wide ducts and numerous dilated acini. The clusters of acini along the posterior border are larger than in the rest of the gland. Figure 3, the gland of a female that was exposed to a pellet for 4 weeks and later developed a tumor, looks like a gland of an untreated female. The acini are not distended and are quite uniformly distributed. Several small hyperplastic nodules are present. Figure 4, the gland of a female that was killed after exposure to a pellet for 8 weeks, shows dilated ducts and acini. In the glands of this group of females, the clusters of acini were about the same size as those in females exposed to a pellet for 4 weeks, but they were more uniformly distributed throughout the gland, though there was still a tendency for larger clumps of acini to be located along the posterior border. Figure 5, the gland of a female that developed a tumor after diethylstilbestrol treatment for 8 weeks, shows clusters of small acini and several hyperplastic nodules. The glands of 31 females that had tumors after exposure to a pellet for 8 weeks were similar to those of

untreated females with tumors, except that the ducts and acini in the glands of the mice which developed tumors within 4 to 6 weeks after removal of the pellet were usually somewhat more dilated than in normal females. Figure 6, the gland of a tumor-bearing female that received continuous diethylstilbestrol treatment, shows greatly dilated ducts and acini, but the number of acini in each cluster was not markedly different from that in the glands of mice killed after treatment for 8 weeks. There was not much variation in the degree of acinar development in the glands of 27 mice treated with diethylstilbestrol for life; the hyperplastic nodules were numerous and often large.

Figure 7, the gland of a C3H × RIII male 3 months old, killed after exposure to a pellet for 4 weeks, shows many end buds and numerous acini along the main ducts. End buds were present in the 35 glands examined, which indicated that growth of the gland was taking place. Most of the acini were in the ventral half of the gland. No photomicrographs are shown of the glands of males killed after exposure to a pellet for 6 or 8 weeks, but examination of the slides revealed that after treatment for 6 weeks the glands had increased in size, end buds were absent, and the acini were more numerous. After 8 weeks of treatment the clusters of acini were larger and were in all parts of the glands; small hyperplastic nodules were observed in only 3 out of the 29 glands examined. Figure 8, the gland of a tumor-bearing male that received continuous treatment, shows wide ducts, clusters of distended acini, and several hyperplastic nodules composed of dilated acini. There was not much variation in the degree of acinar development in the glands of 39 mice that were treated for life. The paraffin sections of tumors often contained, in addition to the tumor itself, some of the adjacent estrogen-stimulated mammary tissue. The dilated acini and small ducts, lined with cuboidal or low-columnar epithelium, contained a substance in the lumen which stained pink with eosin and in many instances was organized into concretions. Figure 9, the gland of a male that had a tumor 2 months after removal of the pellet, shows narrow ducts, very few acini, and hyperplastic nodules. Acini were present in very small numbers, as illustrated, or were entirely absent in the glands of 41 males that developed tumors after treatment for 8 weeks. The glands of males which developed tumors after exposure to a pellet for 4 or 6 weeks were similar to those of males treated for 8 weeks.

Many whole mounts were made of the glands of estrogen-treated male and female mice of the reciprocal cross, RIII × C3H, but descriptions of these would be repetitious as no outstanding differences could be observed in the glands of the two hybrid groups.

DISCUSSION

Andervont, Dunn, and Canter (7) found that subcutaneous implantation of diethylstilbestrol pellets can produce relatively high incidences of

mammary tumors in some strains of mice without the mammary-tumor agent. They stated that a tumor incidence of 84 percent at a mean age of 13 months in castrated, agent-free RIII × C3H males was of special interest, for it showed that mice of this hybrid cross are excellent animals for studies of mammary tumors that develop in the absence of the agent.

There is no genetic difference in the hybrid mice of reciprocal crosses between strains C3H and RIII and therefore the genetic susceptibility to mammary tumors is the same. The difference in the rate of tumor formation in virgin females of the two hybrids suggests that the agent present in the milk of strain RIII mice is less stimulating to early development of mammary tumors than the agent in the milk of strain C3H mice (*5*). Hormonal stimulation, which is the third factor that plays an important role in the development of mammary tumors, may be increased by treating mice with exogenous hormones. When diethylstilbestrol pellets were implanted into females of reciprocal crosses between strains C3H and RIII and left in for life, the mean tumor age was nearly the same in the two hybrids. This showed that the rate of tumor formation was not greatly affected by the difference in the potency of the agent in C3H and RIII. The mean tumor age of C3H × RIII females, exposed to a pellet for 8 weeks, was as low as in females that received treatment for life. In the reciprocal cross, RIII × C3H, tumors did not appear as early in females treated for 8 weeks as in females with continuous treatment. The differences in tumor incidences and rates of tumor formation in the two hybrids were more pronounced in males treated with estrogen for varying periods. C3H × RIII males exposed to a pellet for 6 or 8 weeks developed a much higher percentage of tumors and they appeared at an earlier age than in RIII × C3H males with the same treatment. When the pellets were left in for life, both hybrids had a high incidence of tumors, but they appeared somewhat later in the RIII × C3H males.

One explanation for the relative lack of reciprocal differences in the mean tumor ages of the groups exposed to diethylstilbestrol for life is that there is little room for a reduction in the time that tumors appear in the C3H × RIII cross, whereas there is a large reduction in time possible in the RIII × C3H cross. This explanation might indicate that this is not a true biological interaction of the treatments with the reciprocal crosses (or tumor agents) but a matter of approaching an upper limit of carcinogenesis in the untreated C3H × RIII cross.

Results obtained previously with RIII × C57L hybrids (*4*) showed that approximately 10 percent of the males exposed to a pellet of diethylstilbestrol for 6 or 8 weeks developed tumors at a mean age of 18 months. Virgin females of this cross had a fairly high percentage of mammary tumors (63%), but the mean tumor age was 21 months. In the three hybrid groups studied (C3H × RIII, RIII × C3H, and RIII × C57L) there is a correlation between the susceptibility of males to estrogen treatment for short periods and the rate of tumor formation in untreated virgin females.

The C3H × RIII hybrid is of particular interest because a high per-

centage of mammary tumors appeared at an early age in untreated virgin females and the mean tumor age of females treated with diethylstilbestrol for only 8 weeks was slightly less than in force-bred females. Male mice also provide a source of mammary tumors when treated with diethylstilbestrol. The optimum period of treatment for C3H × RIII males was not established, since only 78 percent of the mice developed tumors after exposure to a pellet for 8 weeks. It seems probable that if the pellets were removed after 10 weeks the tumor incidence would be significantly increased and the mean tumor age lowered. The tumors developing in mice that have received continuous estrogenic stimulation sometimes have soft areas filled with a milky secretion, in contrast to the firm tumors which usually appear after discontinued estrogen treatment. Furthermore, prolonged treatment with estrogen is more liable to cause pathological changes in other organs than when the mice are exposed to pellets of diethylstilbestrol for relatively short periods.

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PLATE 143

All figures are photomicrographs of third mammary glands of (C3H × RIII)F_1 hybrid virgin female mice. × 9

FIGURE 1.—Gland of normal female, 3 months old, showing few lateral buds. Glands of 45 percent of the females in this group were similar to this.

FIGURE 2.—Gland of a female, 3 months old, killed after exposure to a diethylstilbestrol pellet for 4 weeks, shows wide ducts and numerous dilated acini. *Note* large clusters of acini along the posterior border.

FIGURE 3.—Gland of tumor-bearing female, 10 months old, that had previously been exposed to a pellet for 4 weeks. This looks like the gland of an untreated female since the acini are not distended. *Note* several small hyperplastic nodules.

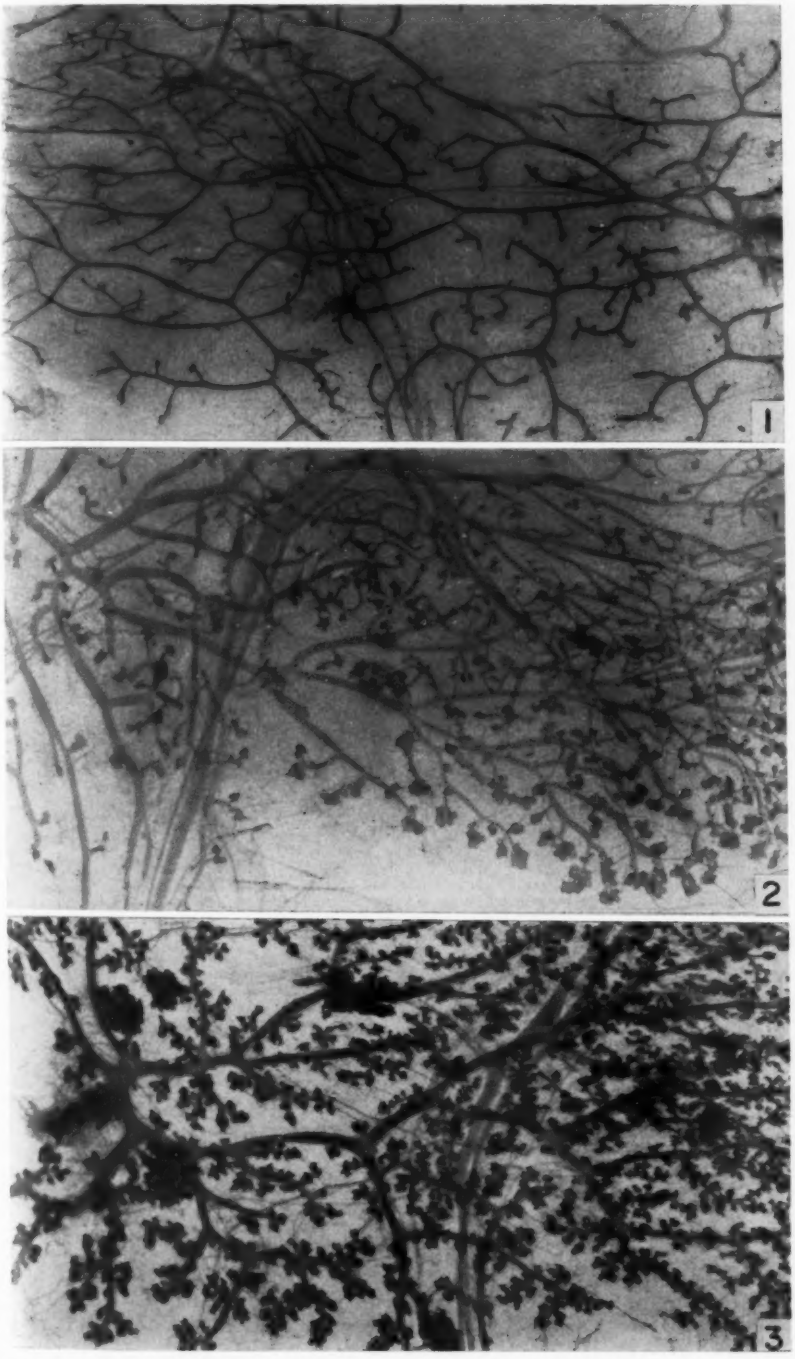
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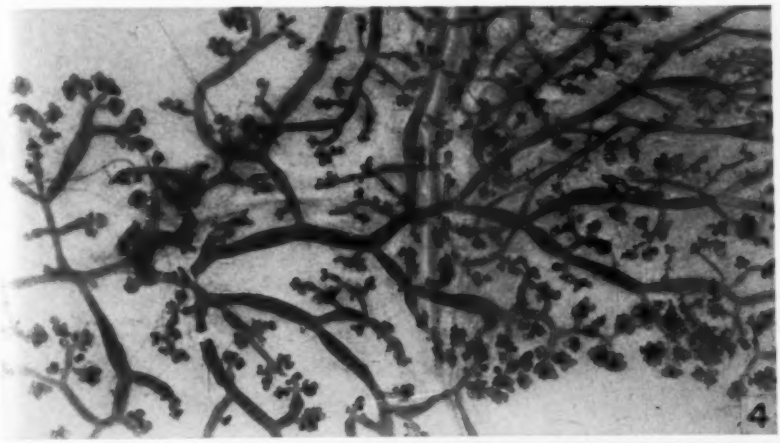
PLATE 144

All figures are photomicrographs of third mammary glands of (C3H × RIII)F_1 hybrid virgin female mice. × 9

FIGURE 4.—Gland of a female, 4 months old, killed after exposure to a pellet for 8 weeks. Ducts are wide and clusters of distended acini are present in all parts of the gland.

FIGURE 5.—Gland of a tumor-bearing female, 7 months old, that had previously received diethylstilbestrol treatment for 8 weeks. *Note* clusters of small acini and several hyperplastic nodules.

FIGURE 6.—Gland of tumor-bearing female, 6 months old, that received continuous diethylstilbestrol treatment. Ducts and acini resemble those shown in figure 4. *Note* large nodule.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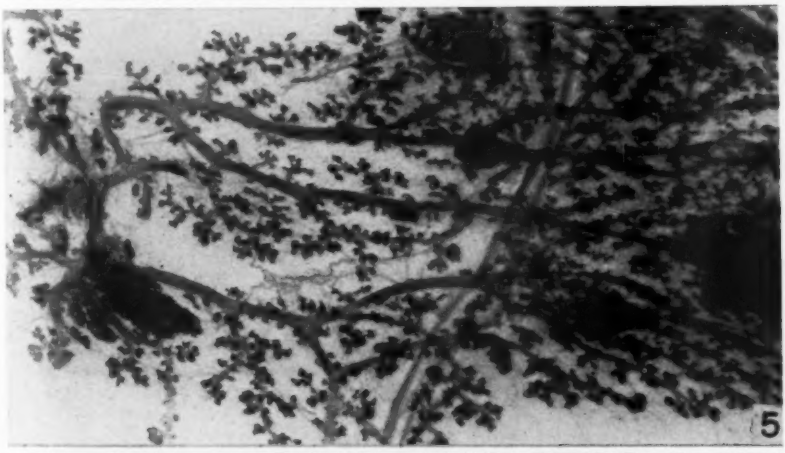
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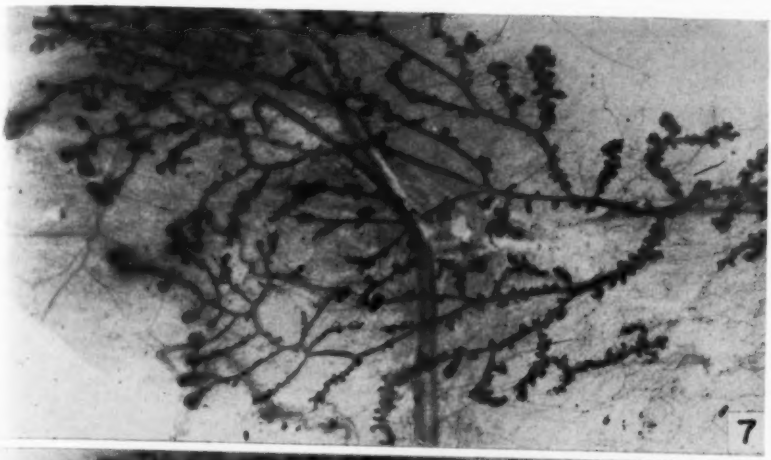
PLATE 145

All figures are photomicrographs of third mammary glands of (C3H × RIII)F_1 hybrid male mice. × 9

FIGURE 7.—Gland of a male mouse, 3 months old, killed after exposure to a pellet for 4 weeks, shows many end buds and numerous acini along the main ducts.

FIGURE 8.—Gland of a tumor-bearing male, 6 months old, that received continuous diethylstilbestrol treatment, shows wide ducts, clusters of distended acini and hyperplastic nodules which vary in size.

FIGURE 9.—Gland of a tumor-bearing male, 6 months old, that had previously been exposed to a pellet for 8 weeks, shows narrow ducts, almost complete regression of acini and small nodules.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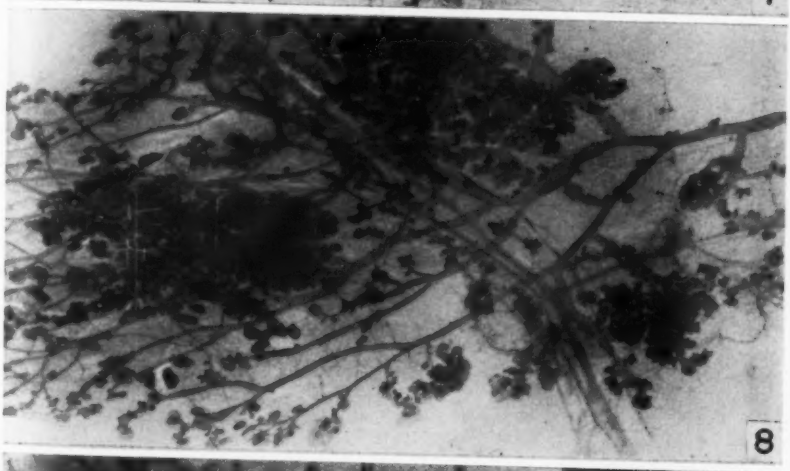
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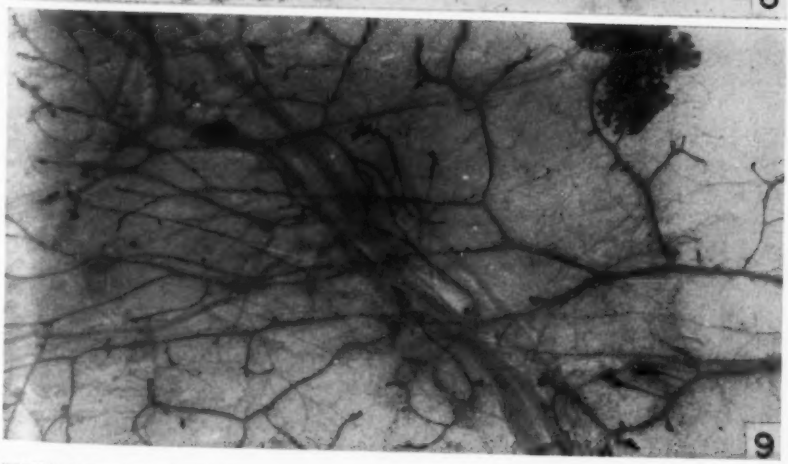
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Relationship Between Chromosome Ploidy and Radiosensitivity in Selected Tumor Sublines of Common Origin [1,2]

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SUMMARY

A predominantly hypertetraploid subline, ELT/stock was derived from a predominantly hyperdiploid Ehrlich ascites tumor, ELD, after homotransplantation of sublethally X-irradiated cells. The ploidy shift was probably the result of immunoselection based on the differential resistance of the polyploid cells against the homograft reaction. Two additional sublines, a pure hypertetraploid, ELT/clone, and a predominantly hyperdiploid population, ELTD, were subsequently selected from ELT/stock. The former was obtained by isolating a hypertetraploid clone (s), and the latter by carrying ELT/stock in serial passage in the absence of immunoselection, in which case hyperdiploid cells regain dominance. The ELTD subline was cytologically indistinguishable from the main ELD line. After irradiation *in vitro* with different X-ray doses, all sublines were grown intraperitoneally within Millipore diffusion chambers with an average pore size of 0.45 μ, thus isolated from direct contact with host cells. The size of cell population was determined quantitatively at various intervals after inoculation. The growth behavior of all untreated sublines was closely similar. There was a difference in their radiosensitivity, however. After the same dose of X rays, the hypertetraploid sublines started to grow earlier and/or reached a larger population size than the hyperdiploid lines.—J. Nat. Cancer Inst. 25: 1041–1063, 1960.

SPONTANEOUS OR induced primary or transplanted neoplasms frequently show a considerable variation in chromosome number both *in vivo* and *in vitro* (*1*, *2*). It is not known whether there is any relationship between the radiosensitivity and the chromosome ploidy of different tumor cells in a heterogeneous population. If we assume that damage to the genetic material plays a dominant role in the cytocidal effect of X rays (*3*, *4*), it is reasonable to expect that polyploid cells may be more resistant than diploids, since they may require the inactivation of a larger number of homologous, sensitive sites. Experiments on plants and fungi

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are consistent with this assumption (*5–7*). No direct extrapolations to neoplastic cells are permissible, however, since the predominant cell type, stem line, is aneuploid in many tumors. Frequently, however, the main stemline number is doubled, more or less exactly, in a smaller second stem line (*1*). Strictly euploid series are infrequently found in tumors. It cannot be predicted how a given aneuploid stem line will react to radiation as compared to the doubled stem line. Rearrangements occurring concurrently with or subsequently to duplication may render sensitive sites monosomic and make the cell more sensitive to radiation.

Since different tumors differ widely both with regard to radiosensitivity and the distribution of chromosome numbers, studies on the relationship between ploidy and radiosensitivity must be carried out with sublines derived from the same original tumor-cell population, which differ in chromosome ploidy *or* in radiosensitivity. Positive observations could be interpreted with more confidence if such material is used than in a comparison of independent tumor-cell populations, differing in many other, not necessarily relevant, respects.

Parallel tumor sublines of common origin differing in chromosome ploidy have been developed by Kaziwara (*8*) and by Hauschka *et al.* (*9*) from the Ehrlich ascites tumor and the 6C3HED ascites lymphoma, respectively, utilizing the differential sensitivity of tumor cells with different chromosome numbers to the homograft reaction. A similar procedure led to the establishment of a hypertetraploid subline of the hyperdiploid Ehrlich ascites tumor at our laboratory (*10*).

Several attempts to demonstrate a difference between the radiosensitivity of the hyperdiploid and hypertetraploid cell populations gave negative or equivocal results. With increase of cellular mass in the absence of mitosis as a measure of radiosensitivity, both sublines were equally radioresistant after treatment with 1250 r (*10*). Inconsistent and often contradictory results were obtained in experiments *in vivo*, measuring mitotic recovery or determining the total cell number after different X-ray doses (Révész, unpublished data). The poor reproducibility of these experiments may have been due to the immunizing action of the irradiated cells upon their immunogenetically foreign, homologous hosts (*11*). The strength of the homograft reaction is an unknown variable, superimposed upon the inherent radiosensitivity of the graft (*11–13*).

The present work represents an attempt to study the relationship between radiosensitivity and chromosome ploidy in an *in vivo* system where the interference by the homograft reaction could be excluded. Irradiated sublines of the same original ascites tumor, differing with regard to their modal chromosome number, were grown intraperitoneally within diffusion chambers. This technique (*14*) permits the isolation of the graft from direct contact with host cells. Filter membranes with appropriate pore size permit the diffusion of soluble material, but prevent the inward passage of the host cells and the outward movement of the graft cells. Homografts of various kinds have been shown to survive and to proliferate for long periods in diffusion chambers (*14–16*). Recently,

methods have been developed to estimate quantitatively the size of the cell population within the chamber (*15*, *17*).

MATERIALS AND METHODS

Mice.—Various F_1 hybrids were used, obtained by mating males of the A.SW/Sn/Kl strain with females of the strains C3H/Kl, DBA/2/Kl, and C57BL/Kl. The animals weighed 20 to 26 gm. and were 2 to 4 months old. They were maintained on a standard pellet diet, which, with drinking water, was available *ad libitum*.

Tumor.—An Ehrlich ascites tumor line, also known under the misleading synonym Landschütz Sarcoma I (*18*), or under the designation LE (*8*), was used. The main line of this tumor will be referred to as ELD (*10*). Three derived sublines will be designated as ELT/stock, ELT/clone, and ELTD, respectively. The history and characteristics of the 3 sublines are described under "Results." The chromosomal characteristics of ELD have been studied in detail (*18*, *19*). The majority of the cells are hyperdiploid, and cells with a doubled, hypertetraploid stemline number occur in 1 to 5 percent. The growth characteristics of ELD and ELT/stock *in vivo* have been investigated by Hauschka *et al.* (*10*).

ELD and its sublines were maintained in adult, heterozygous mice by serial intraperitoneal transfer of 0.1 ml. ascites, diluted tenfold with Ringer's solution, containing 100 I. U. penicillin and 100 μg. streptomycin per ml. During several periods of varying length, the cells were preserved by frozen storage at −79° C. in a tumor bank as described previously (*20*). The cell suspensions used in the actual experiments were always derived from ascites tumors that had grown in the peritoneal cavity for 8 to 12 days. Appropriate dilutions were made with sterile Ringer's solution.

Cellular characteristics.—The *proportion of tumor cells* in the ascitic fluid was determined by differential counting of tumorous and nontumorous cells on smears prepared according to the method of Papanicolaou (*21*). Four hundred cells were counted on each slide.

For *chromosome counting*, tumor-bearing animals received intraperitoneal injections of 0.5 ml. of a 0.04 percent Colcemid (Ciba Pharmaceutical Products, Inc., Summit, N.J.) solution. Two hours after the injection ascitic fluid was withdrawn, treated for 20 minutes with 1.12 percent sodium citrate, and fixed in a 3:1 mixture of ethanol and acetic acid. Subsequently, aceto-orcein squashes were prepared on coverslips. Cells with a doubled chromosome set could be easily distinguished from hyperdiploid cells by scoring 50 to 100 well-spread metaphase plates at a magnification of 600× with a Zeiss standard phase contrast microscope. The approximate frequency of hypertetraploid cells was determined by this method in all inoculated samples in each experiment. In addition, exact chromosome counts were made for all tumor lines on drawings

prepared with an apparatus of Abbe's type and at a magnification of 1500×. Only cells with intact border lines were included.

The *cellular mass* of the packed ascites cells and their *average volume* were determined by the hematocrit method in combination with cell counting (*22*).

The *average amount of deoxyribonucleic acid* (DNA) per cell was determined by the method of Schneider (*23*), in aliquots of the undiluted ascitic fluid containing known cell numbers.

Diffusion-chamber technique.—Diffusion chambers of the previously described type were used (*15*). They were constructed of acrylic rings and Millipore filters, HA type (Millipore Filter Corp., Bedford, Mass.) with a pore size of 0.45 ± 0.02 μ and had a volume of 0.4 ml. (height 2.5 mm., diameter 14.5 mm.). Their sterilization was described previously (*15*).

Diluted suspensions containing 8×10^6 untreated or irradiated cells per 0.2 ml. were introduced in 0.2 ml. amounts into the chambers, unless otherwise stated. Within each experiment, 10 to 20 chambers were filled and subsequently introduced into the peritoneal cavity of mice, anesthesized with 10 mg. per kg. Nembutal, and administered intraperitoneally in a 0.9 percent saline solution. At regular intervals after the operation, 2 to 3 animals of each series were killed by cervical dislocation. The chambers were removed and their cellular contents determined quantitatively. The procedure of introducing the cells into the chambers and the technique of quantitative sampling have been published elsewhere (*15*).

The variability involved in the determination of the total-cell number in different chambers within the same experiment was calculated from parallel counts on duplicate and triplicate chambers inoculated and removed in the same way and at the same time. Based on a total of 16 duplicate and 24 triplicate determinations chosen at random from all experiments, a standard deviation of 0.0131 in log units was calculated for the means of the duplicates and triplicates, respectively. The means themselves were distributed between 6.5 and 7.5 log units. This standard deviation is in good agreement with similar data obtained with a different ascites tumor (*15*).

Repeat experiments were carried out to check the reproducibility of the values and to estimate the variability involved. A total of 17 duplicate and 6 triplicate values was analyzed. These were obtained in two or three *separate* experiments at corresponding times; each value was, in turn, based on duplicate or triplicate determinations *within* each experiment. The standard deviation between experiments was equal to 0.0374 in log units. Thus the variability between repeat experiments, due to such factors as differences in the tumor material, errors of inoculation, cell counting and dilution, differences in the radiation conditions, etc., was considerably larger than between duplicate chambers within the same experiment.

Irradiation.—Ice-cooled tumor-cell suspensions, diluted with Ringer's

solution to a concentration of 40 × 10^6 cells per ml., were irradiated in a sterile, flat-bottomed Perspex irradiation chamber. The vial had an inside diameter of 26 mm. and a height of 24 mm. and was equipped with a removable lid. After 5 ml. of the cell suspension was introduced, the vial was placed under the X-ray tube 30 minutes before irradiation.

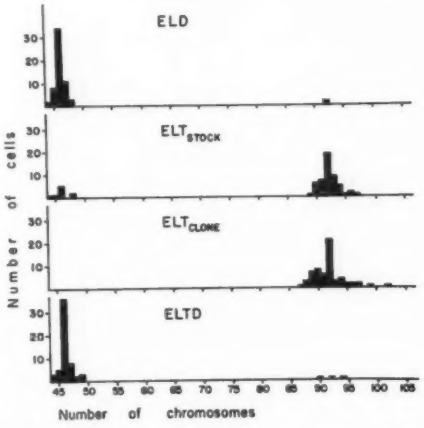
X rays were generated in a Siemens' X-ray machine at 185 kv. and 15 ma. and were filtered by 1 Al. The dose rate was 425 r per minute at the bottom of the vial at a distance of 29.5 cm. from the focus.

RESULTS

Derivation of Tumor Sublines Differing in Chromosome Ploidy

The ELD tumor remained unchanged with regard to its modal chromosome number and its structurally altered marker chromosomes since the studies of Bayreuther (*19*), Tjio and Levan (*18*), and Kaziwara (*8*). The cell population consisted predominantly of hyperdiploid cells (*see* col. 2 of table 1 and text-fig. 1). The modal stemline number was 46. The frequency of cells with a doubled stemline number was less than 5 percent. The markers included one chromosome with a secondary constriction, designated "A" by Bayreuther (*19*), one metacentric unit, called "B," and 2 or 3 "minutes." A persistence of chromosomal characteristics has also been reported by Hauschka (*24*) in another line of the ELD tumor in the course of 153 serial passages during 3½ years.

Three sublines were derived from ELD, denoted ELT/stock, ELT/clone, and ELTD, respectively. The establishment of these sublines was based on Kaziwara's method of "immunoselection" (*8*), leading to cell populations differing in their modal chromosome number.



TEXT-FIGURE 1.—Distribution of chromosome numbers in 60 cells of the ELD, ELTD, ELT/stock, and ELT/clone lines.

TABLE 1.—Properties of the four sublines of the Ehrlich ascites tumor
(*n = number of samples examined*)

Property	Tumor line			
	ELD	ELTD	ELT/stock	ELT/clone
1	2	3	4	5
Frequency of tumor cells in the cell population	92% (n = 39)	89% (n = 6)	91% (n = 33)	92% (n = 5)
Proportion of metaphase plates in the hypertetraploid region	<5% (n = 7)*	<5% (n = 5)*	>85% (n = 6)*	100% (n = 7)*
Modal chromosome number:				
(*a*) Own counts	46 (n = 2)†	46 (n = 2)†	92 (n = 1)†	92 (n = 2)†
(*b*) Counts from the literature (*17, 18*)	46			
Structurally changed chromosomes:				
Minutes	2–3	2–3	4–6	4–6
Secondary constriction	1	1	2	2
Metacentric chromosome	1	1	2	2
Average cell volume	1386 ± 47 μ^3 (n = 25)	1370 ± 51 μ^3 (n = 6)	2238 ± 67 μ‡ (n = 27)	2611 ± 58 μ^3 (n = 5)
Packed cell volume	32% (n = 25)	31% (n = 6)	36% (n = 27)	31% (n = 5)
Concentration of free cells per ml. ascitic fluid	232 ± 12 × 10^6 (n = 39)	226 ± 16 × 10^6 (n = 11)	164 ± 9 × 10^6 (n = 33)	119 ± 9 × 10^6 (n = 10)
Average DNA content per cell	11.1 ± 0.5 × 10^{-9} mg. (n = 5)		20.6 ± 1.1 × 10^{-9} mg. (n = 6)	24.2 ± 4.6 × 10^{-9} mg. (n = 12)

Average extinction per Feulgen stained cells at 5460 Å (relative units)‡		
Metaphase cells	25 (n = 1)§	54 (n = 1)§
Interphase cells	21 (n = 1)§	47 (n = 1)§
Average duration of mitosis ‖	30 minutes	60 minutes
Maximum total-cell number ‖	2×10^9	1×10^9
Maximum total neoplastic mass ‖	2×10^3 mm³.	2×10^3 mm³.
Increase of average cell volume per hour after irradiation in the absence of mitosis ‖	15.3 μ³ (1.37%)	31.6 μ³ (1.38%)

*In each sample 50 to 100 metaphase cells were scored.

†The chromosomes of a total of 60 metaphase cells were counted exactly in the samples. The distribution of chromosome numbers is illustrated in text-figure 1.

‡According to reference (*25*).

§One hundred cells were measured in each sample.

‖According to reference (*10*).

(1) The ELT/stock subline originated from 1 of 8 mice inoculated intraperitoneally with 20×10^6 ELD cells that had been irradiated with 4000 r *in vitro* in an N_2 atmosphere. As will be discussed later, conditions may be especially favorable for immunoselection after homotransplantation of sublethally irradiated cells. Four mice died within 2 months with ascites tumor and 3 remained entirely free of tumor during an observation period of 6 months. In 1 animal a solid tumor appeared after 4 months and infiltrated the peritoneal cavity. Cells of this tumor were suspended in Krebs-Ringer phosphate, and 10^6 cells were inoculated into the peritoneal cavity of 10 mice. All animals developed ascites containing a predominantly hypertetraploid tumor-cell population. The ascites tumor of one mouse was preserved in a series of tubes in our frozen tumor bank. The designation "ELT/stock" refers in the present paper exclusively to the first 3 consecutive animal passages of the thawed material from these tubes. The stem line of ELT/stock appeared to be an exact duplication of the ELD stem line (*see* col. 4 of table 1 and text-fig. 1). The chromosomal markers of ELD were present in a double dose (*see* figs. 1 and 2 of plate 146). Hyperdiploid cells were found in less than 15 percent. The growth characteristics of the ELT/stock line have been described previously (*10*). Data pertinent to the present investigation are shown in column 4 of table 1. These are in agreement with the results of others on analogous materials (*8*, *10*, *26*, *27*).

(2) The ELTD subline was derived from ELT/stock by carrying it in serial passage with high inoculum doses (20×10^6 cells for 12–15 transplant generations). In agreement with the findings of Kaziwara (*8*), the mixed population shifted back gradually from hypertetraploid to hyperdiploid in the absence of immunoselection. The ELTD subline was indistinguishable from the main ELD line with regard to its cytological properties (*see* col. 3 in table 1).

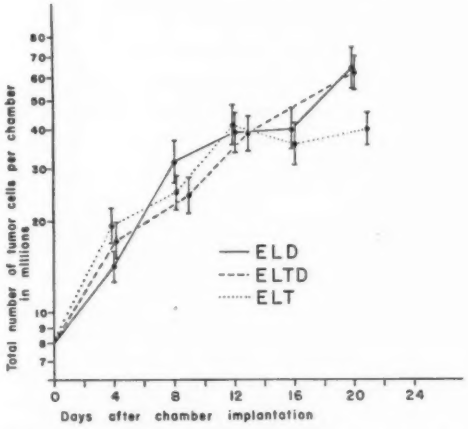
(3) To obtain a pure and stable hypertetraploid population, experiments were performed to isolate a hypertetraploid clone. Aliquots from two separate ELT/stock populations were diluted $1:15 \times 10^6$ and $1:17 \times 10^6$, respectively, in Krebs-Ringer phosphate containing 0.25 percent gelatin. Subsequently, 0.1 ml. volumes calculated to contain one cell on an average were inoculated into the abdomen of 70 newborn, hybrid mice. Tumors developed in a total of 9 animals during an observation period of 45 days after inoculation. One part of each tumor was preserved in our frozen tumor bank; another part was diluted tenfold and transplanted intraperitoneally in 0.1 ml. volumes into groups of 3 to 4 mice. All inoculated animals developed ascites tumors. The tumors of two groups contained cells with metaphase plates in the hypertetraploid region only, while the remaining 7 groups developed predominantly hyperdiploid tumors. The frozen material corresponding to one of the hypertetraploid populations was thawed and, denoted as ELT/clone, was carried serially by intraperitoneal transfers of 0.1 ml. ascites at 10-day intervals. In the course of 14 successive transplant generations the modal chromosome number of this line remained stable at 92, and the cells contained a duplicate set of

the markers. No hyperdiploid metaphase plates were observed (*see* col. 5 in table 1 and text-fig. 1).[5]

Radiosensitivity Tests

In a series of experiments the multiplication of 8×10^6 untreated or irradiated cells of the ELD, ELTD, ELT/stock, and ELT/clone lines was studied in diffusion chambers. The results obtained with the two latter lines were pooled and will be referred to collectively as observations on the ELT lines.

Text-figure 2 shows the growth curves of the untreated cells during a period of 20 days. The cells of all 3 lines started to increase in number soon after inoculation. Within 20 days ELD and ELTD reached a mean population size of about 60×10^6 cells. During the same period ELT increased to about 40×10^6 cells per chamber. The final ELT population was thus lower than ELD. This was previously found to be true when the cells were permitted to multiply freely in the abdominal cavity (*10*): Cell proliferation came to a standstill when the populations reached the size of 1×10^9 (ELT) and 2×10^9 (ELD) cells per mouse, respectively.

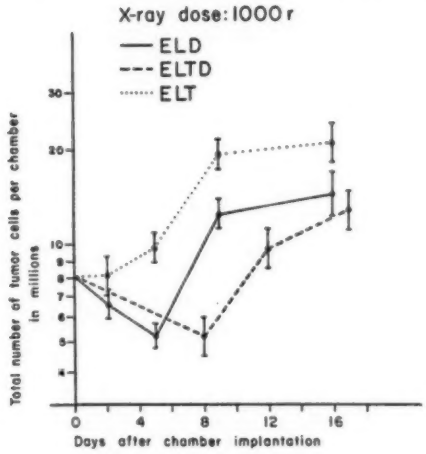


TEXT-FIGURE 2.—Growth of untreated hyperdiploid ELD, ELTD, and hypertetraploid ELT in diffusion chambers. The geometrical mean and the standard error are indicated. The means have been calculated from 2 to 5 separate chambers in 1 or 2 separate experiments. The standard errors are based on 17 duplicate and 6 triplicate determinations.

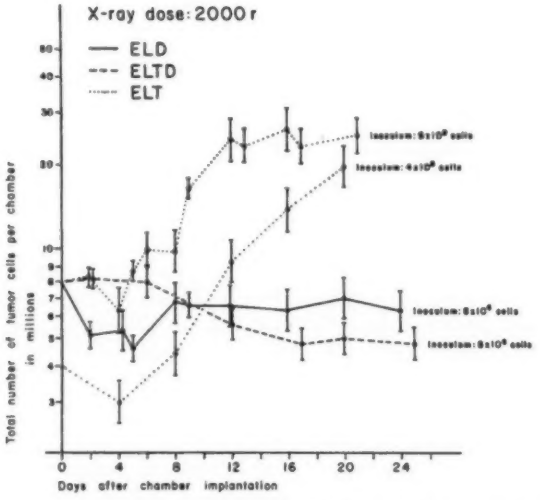
Text-figures 3 to 6 illustrate the behavior of irradiated cells. After a dose of 1000 r, all 3 lines showed a certain growth (text-fig. 3). At corresponding times, the total number of ELT cells was, however, consistently larger than that of ELD or ELTD. After treatment with 2000 r, there was no detectable increase of the ELD or ELTD populations during

[5] The ninth transplant generation of the ELT/clone was examined by Dr. A. Levan, Lund, Sweden, who described it as an exact duplication of the ELD line (personal communication).

an observation period of 24 days (text-fig. 4), and a certain progressive decrease could be discerned. Contrariwise, the corresponding ELT inoculum showed a threefold increase. An increase occurred even when the number of inoculated ELT cells was decreased by 50 percent to bring it to the level of the cell mass contained in the hyperdiploid inoculums;

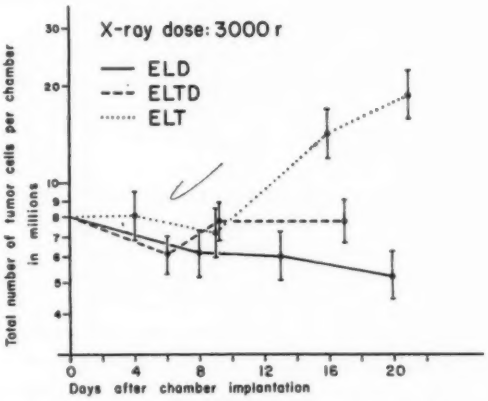


TEXT-FIGURE 3.—Growth curves of the ELD, ELTD, and ELT tumor lines after treatment with 1000 r. Means were calculated from 2 to 7 chambers in 1 or 2 separate experiments.

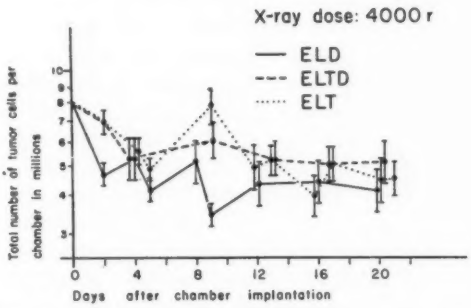


TEXT-FIGURE 4.—Growth curves of ELD, ELTD (inoculum dose, 8×10^6 cells), and ELT (inoculum dose, 8×10^6 and 4×10^6 cells, respectively) after treatment with 2000 r. The means were obtained from calculations based on 2 to 10 chambers in 1 to 3 separate experiments.

the increase was fivefold in this case. The ELT line was not completely inhibited by 3000 r X rays; the irradiated cells doubled their number by the end of the 3d week (text-fig. 5). No growth occurred with ELD and ELTD. Irradiation with 4000 r (text-fig. 6) or with 8000 r prevented growth completely and the cell numbers decreased at about the same rate with all 3 lines. About 50 percent of the original cell number could be still recovered 20 days after inoculation, which indicated that dead cells persisted in the chamber. A similar finding has been made even after irradiation with 15,000 r (*15*).

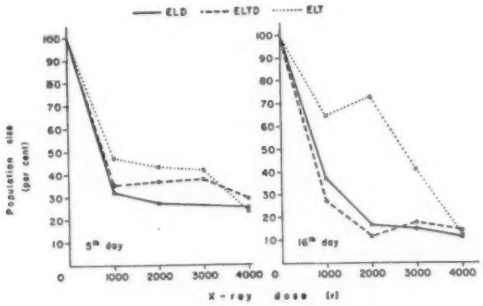


TEXT-FIGURE 5.—Growth curves of ELD, ELTD, and ELT lines after treatment with 3000 r. The means were obtained from 2 to 3 chambers in 1 or 2 separate experiments.



TEXT-FIGURE 6.—Cell content of diffusion chambers after inoculation of ELD, ELT, and ELTD treated with 4000 r. The means were obtained from 2 to 6 chambers in 1 to 3 separate experiments.

A comparison of the growth curves of untreated and irradiated ELT, ELD, and ELTD cells, respectively, shows that all irradiated populations were consistently smaller than corresponding untreated populations. The relationship between the size of the untreated and irradiated lines after 5 and 16 days of growth is illustrated in text-figure 7.



TEXT-FIGURE 7.—Effect of different doses of X rays on the population size of ELD, ELT, and ELTD 5 and 16 days after the inoculation of 8×10^6 cells. The points represent the population size as percentage of corresponding controls.

DISCUSSION

In studies on the radiosensitivity of tumors it is of particular importance to distinguish between the effects that can be ascribed to the radiation energy absorbed within the tumor cells themselves and effects mediated by the irradiated host environment. The latter type of influence, usually referred to as "indirect radiation effects," has been recently reviewed by Scott (*28*). In experiments involving the transplantation of irradiated tumors into untreated recipients, indirect effects resulting from radiation damage to the tumor bed, the blood vessels, etc., and consecutive local and systemic repair processes can be avoided. Transplantation of the irradiated cells may provoke local or systemic response in the host which may influence tumor growth in various ways. Inflammatory reaction, accelerated stroma formation, and increased vascularization were shown to occur locally at the transplantation site (*29*), and they may exert a stimulating action on tumor growth (*29*). Such influences may interfere with the testing of cellular radiosensitivity and may lead to a spurious impression of radioresistance. In tumor-host systems where an immunogenetical difference exists between the graft and the recipient, the homograft reaction may be bolstered by the irradiated cells, which may result in an inhibition of tumor growth (*11*). For this reason, the regression of a *homo*transplanted, irradiated tumor does not necessarily indicate that lethal radiation damage has been inflicted on all cells, or even the majority of the cells. This point has been also emphasized by the experiments of Cohen and Cohen (*12*), and Goldfeder (*30*). They consider the radiosensitivity of transplanted tumors in genetically different hosts as a measure of subliminal host resistance which results from immunogenetical differences in the tumor-host relationship. These considerations and the wish to exclude host influences of these kinds from the testing of radiosensitivity of neoplastic cells have motivated the present approach, with the use of the diffusion-chamber technique of Algire *et al.* (*14*). When

filter membranes with an adequately small pore size are applied, this technique permits the isolation of a preirradiated graft from a direct contact with the host tissues. Grafts of various kinds were shown to survive and proliferate for long periods within such chambers (*14–16*).

In many tumor-cell populations the modal chromosome number remains unchanged in the course of serial transfer. This was regarded as evidence for the continuity of the "stemline" cells (*2, 31–33*), and an indication of their importance as "the principal contributors to the growth of the tumor" (*2*). An experimentally induced shift of the stemline number was accomplished by Kaziwara in 1954 (*8*). He inoculated small numbers of viable cells from the predominantly hyperdiploid Ehrlich ascites tumor into adult Swiss mice. A shift occurred in favor of the originally small minority of hypertetraploid cells. This was interpreted as an "immunoselection" (*8, 9*) operating on the basis of increased resistance of hypertetraploid cells against the homograft reaction, as compared to the hyperdiploid cells. The inoculation of small cell numbers permitted a more prolonged exposure of the cell population to the homograft reaction than the usual technique of inoculating large cell numbers that grow and kill rapidly. A similar mechanism was probably operating in our system in which the hypertetraploid ELT/stock line arose after the inoculation of ELD cells irradiated with 4000 r. This dose damages the majority of the irradiated cells in an irreversible way. Such lethally damaged cells can strengthen the homograft reaction considerably (*11*) and increase its effect on the small, viable remainder of the population.

Unless special precautions are taken and selection pressure is applied consistently, the "immunoselected" but mixed tumor-cell population with a hypertetraploid majority and a hyperdiploid minority will gradually revert toward the predominance of the hyperdiploid cells after 5 to 10 passages (*cf. 8, 26, 27*). In the absence of selection, hyperdiploid cells thus have a proliferative advantage over hypertetraploid cells. This can be explained by a consideration of the growth curves of these lines (*10*). Inoculation is usually performed during the later stages of growth; since cellular mass and not cell number is the factor that limits cell multiplication during the later part of the growth cycle, hyperdiploid cells, originally present in the population, can be expected to increase their relative frequency before each transfer. Indeed, the hyperdiploid ELTD population established after 15 serial transfers of the hypertetraploid ELT/stock line with large cell doses was quite similar to the original ELD population (*cf.* table 1). This backward shift could be efficiently prevented and the population stabilized by establishing a hypertetraploid single-cell clone. The mixed population can be maintained only with a hypertetraploid dominance if subjected to immunoselection upon each passage (*8*).

The ratios of mean chromosome number, average cell volume, and average amount of DNA per cell were approximately 1:2 when ELD and ELT/stock or ELT/clone were compared (*cf.* table 1). The growth characteristics of the hyperdiploid and hypertetraploid lines were closely

TABLE 2.—Survey of studies concerned with chromosome number and X-ray sensitivity
(*n* = *haploid value*)

Material	Parameter studied	Radiation sensitivity	Reference
Seeds of *Avena* *Triticum*	Frequency of chlorophyll mutations	2n>4n	(*34*)
Seeds of *Hordeum*	Germination, seedling growth	2n>4n	(*35*)
Seeds of *Triticum*	Germination, sprouting ability	2n>4n	(*36*)
Seeds of *Triticum* *Avena* *Hordeum* *Secale cereale* *Zea mays*	Germination, seedling height	2n>4n>6n	(*37*)
Seeds of *Triticum*	Seedling growth	2n~4n>6n	(*38*)
Seeds of *Triticum* *Avena* *Hordeum*	Frequency of chromosome aberrations per cell	2n<4n<6n	(*39*)
Root tips of *Triticum* *Bromus*	Inhibition of mitosis	2n>4n>6n	(*40*)
Plants of *Chrysanthemum*	Survival on field	2n>4n>8n>12n>22n	(*5*)
Plants of *Sedum*	Survival on field	4n>·>·>16n	(*5*)

Microspores of *Tradescantia*	Frequency of chromosome aberrations per cell	n∼2n	(*41*)
Microspores and pollen grains of *Tradescantia*	Frequency of chromosome aberrations per cell	n<2n	(*42*)
Microspores of *Tradescantia*	Frequency of chromosome aberrations per cell	n<2n	(*43*)
Neurospora conidia (nutritional mutants)	Reproduction	Mononucleate>binucleate	(*7*)
Saccharomyces cerevisiae	Colony formation	n>2n	(*6, 44*)
Saccharomyces c.	Colony formation	n>2n∼4n>3n	(*45*)
Saccharomyces c.	Colony formation	n>6n>4n>3n>2n	(*46*)
Saccharomyces c.	Division rate	n<2n∼4n	(*47*)
Embryos of *Habrobracon*	Hatchability and eclosion	Cleavage stage: n<2n immediate after cleavage: n∼2n Larval stage: n>2n	(*48*)
Embryos of *Drosophila*	Survival and morphological abnormalities	2n∼3n	(*49*)

similar both in the peritoneal cavity (*10*) and within the diffusion chamber. They differed clearly with regard to their response to radiation. ELT/stock or ELT/clone was less sensitive than ELD. After the same dose of X rays, hypertetraploid cells started to grow earlier and/or attained a larger population size than ELD during the same period.

After the difference between ELD and ELT/stock or ELT/clone had been found, the question arose whether the difference in radiosensitivity was directly related to the ploidy difference of these lines, carried independently for a considerable period, or was merely coincidental. A new hyperdiploid line was therefore established (ELTD) by the previously mentioned procedure, which permitted the mixed ELT/stock population to shift back to the predominance of hyperdiploid cells in the absence of selection. Concurrently, radiosensitivity changed from the ELT to the ELD level. This indicates that a difference exists between the radiosensitivity of the hyperdiploid and hypertetraploid cells of the original population and is not coincidental to the separate maintenance of the two sublines.

Studies concerned with the relationship between chromosome number and radiosensitivity have been carried out previously on various biological materials such as plants, fungi, and arthropods. Radiosensitivity was judged according to many different criteria (table 2). If consideration is restricted to cases in which the effect on cell multiplication was studied, radiosensitivity was usually greatest in the haploid cell and decreased with increasing ploidy. This decrease was not regularly progressive in yeast cells (*46*), while in some plants the radiation sensitivity could be calculated to decrease with slightly more than 50 percent for each duplication of the chromosome number (*5*).

The relationship between ploidy and radiosensitivity has been regarded as an important argument favoring the view that damage to the genetic material is a major cause of radiation death (*4*). Whether it was assumed that the sensitive nuclear site(s) was hit by ionizing radiation directly or indirectly through radiation-induced radicals, the survival curves of yeast cells could be interpreted as reflecting the lethal effect of a combination of recessive and dominant mutations (*46*). The probability of survival, in spite of recessive defects, would increase with ploidy, while the opposite would be true for dominant lethal mutations. However, variations of radiosensitivity during the cell-division cycle of *Tradescantia* (*42*), during the budding of yeast cells (*50*, *51*) or at different developmental stages of *Habrobracon* (*48*), suggest that, even though death and ploidy are related, other physiological factors may enter into the picture, superimposing their effect on the genetic damage (*52*).

In other studies the frequency of chromosome breaks *per cell* was found to be roughly proportional to the chromosome number in irradiated cells, while the aberration frequency *per chromosome* was the same in cells with different ploidy (*36*, *37*, *39*, *42*, *43*). However, the opposite finding has also been reported, indicating less chromosome damage in polyploids than in diploids (*41*). Investigations dealing with other parameters

showed that mitotic inhibition by X rays was inversely proportional to the number of chromosomes in certain plants (*40*). In yeast, the length of X-ray-induced division arrest was directly related to the degree of ploidy (*47*). Chromosome breakage and mitotic delay thus failed to show the same correlation with ploidy as radiation effect on cell multiplication, which suggested that the latter effect is not only or primarily due to these types of damage (*50*, *53*).

The chromosome number and DNA content of many animal and human neoplasms show a wide variation (*1*, *2*, *54–57*). Little is known about the possible relationship between chromosome number and radiosensitivity in human tumors. The question arises whether selection of polyploid cells may contribute to the development of radioresistance in irradiated tumors. Cusmano (*58*) and Koller (*59*) reported observations favoring this possibility. Recently Richards and Atkin (*60*) measured that DNA content of tumor cells in biopsied human uterine tumors before, during, and after treatment by intracavitary radium or radiocobalt. Before irradiation, the frequency histogram of DNA values of two different tumors indicated the presence of cells with a wide range of ploidy between diploid and octoploid values. More than 70 days after treatment there was a new distribution of DNA values with a definite mode in the triploid region, which suggested that the tumors now mainly consisted of one cell type, probably representing the progeny of the most resistant cell strain. These recurrences were found to be extremely radioresistant and did not respond to further radiation treatment.

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PLATE

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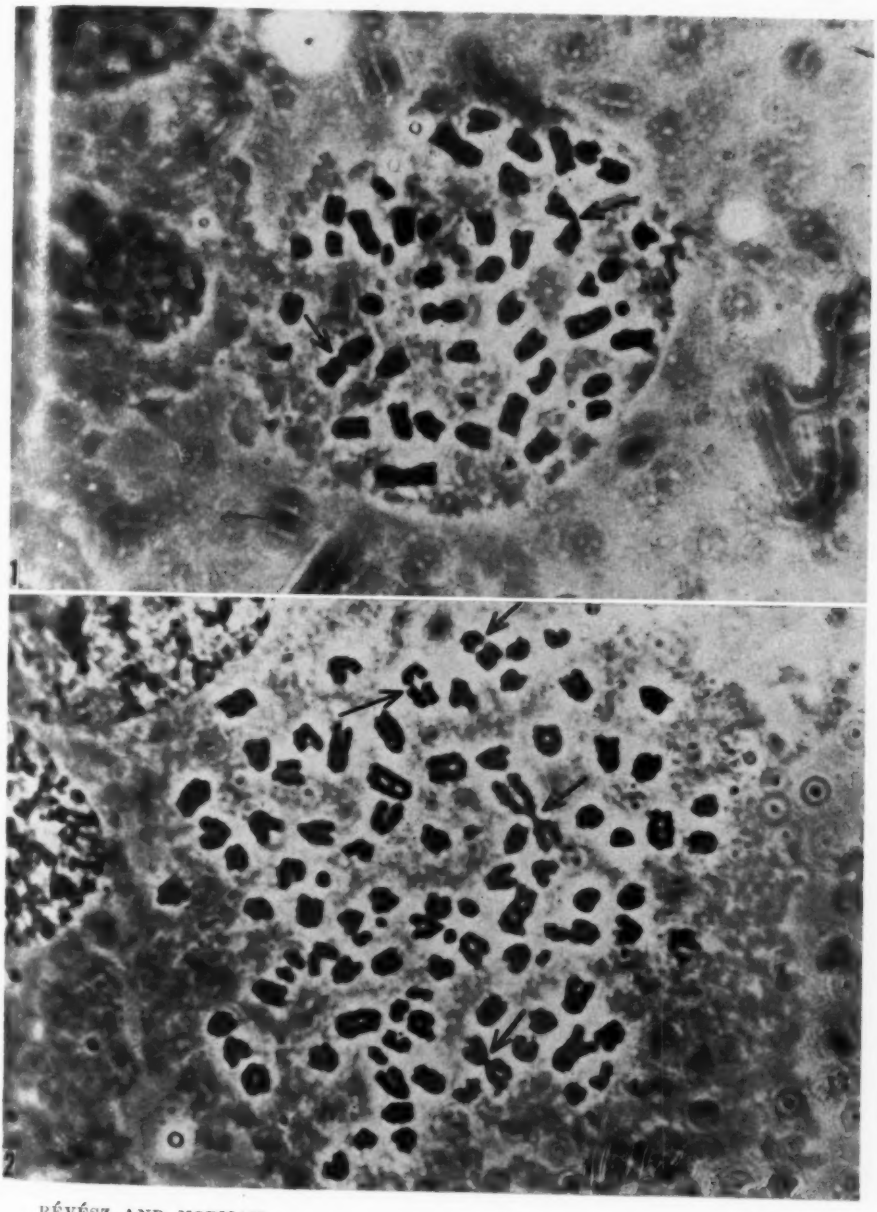
FIGURE 1.—Metaphase of an ELD ascites tumor cell with 47 chromosomes, including 1 metacentric marker, 1 acrocentric chromosome with a secondary constriction, and 3 minutes. The large marker chromosomes are indicated by *arrows*. Aceto-orcein squash preparation. Magnification about × 1,500

FIGURE 2.—Metaphase of a cell from the ELT/clone with 90 chromosomes, including 2 metacentric markers, 2 acrocentric chromosomes with a secondary constriction, and 6 minutes. The large marker chromosomes are indicated by *arrows*. Magnification about × 1,500



Note on the Catalase Activity of Several Mammalian Cell Strains After Long Cultivation *In Vitro* [1]

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SUMMARY

The activity of the enzyme catalase was examined in 12 mammalian cell strains cultured for long intervals *in vitro*. Catalase activity of all cell strains was very low. Certain of the cultured cells were implanted into mice of the inbred strain from which the cells originated. The tumors arising from the implants, both of the sarcoma-producing strains and the one hepatoma-producing strain, had very low activity. The hepatoma-producing strain had much less activity than did spontaneous primary hepatomas excised directly from the mouse.—J. Nat. Cancer Inst. 25: 1065–1068, 1960.

IT WAS reported from this laboratory in 1943 (*1*) that mouse fibroblasts grown for long intervals under *in vitro* culture conditions gave rise to sarcomas at the site of injection when the cells were returned to the mouse strain of origin. This tumor production occurred whether the cells had been treated in culture with 3-methylcholanthrene or not. The malignant change in the absence of added known carcinogen has been amply confirmed and extended in comprehensive serial experiments not only for fibroblasts (*2*) but also for liver parenchymal epithelial cells (*3*). These observations immediately raised the question as to whether culture conditions for cells outside the body could promote the transition of normal fibroblasts to tumor-producing cells during cultivation. To follow changes in cells adapted to growth *in vitro*, the activity of the enzyme catalase was selected as one attribute that could be followed easily with small quantities of cells. When this investigation was started the cell strains available had been in culture for some time, so that this report concerns only well-established cell strains and can be used only for background for changes taking place as the cells gradually accommodate themselves to culture conditions. The fact that three of the long-term cell strains in the laboratory were derived from the parenchymal epithelium of the mouse liver was of prime interest. It is known that liver cells excised directly from the healthy mouse have a high catalase activity (*4*).

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

MATERIALS AND METHODS

The cell strains used, the manner of cultivation, enumeration of nuclei, and sampling have been recorded (*1–3*, *5–8*). The catalase activity of whole-cell homogenates was measured by the method of Sumner and Dounce (*9*). Total protein content of the homogenates was ascertained by the method of Lowry *et al.* (*10*). Aliquots of the normal or tumor tissue taken directly from the animal were examined for catalase activity for comparison with the cultured cells and were treated in similar fashion for assay. The animals were maintained on Purina laboratory chow, and all were 1 year of age or older.

RESULTS

The results for the catalase activity of the several cell strains are listed in table 1. For the convenience of the reader, data on the tissue of origin of the cell strain and the years in culture are included. The enzyme activity of tumors arising from implantation of certain of the cultured cells is listed in table 2. The catalase activities of livers excised from these tumor-bearing mice are likewise listed, and are compared with the livers of non-tumor-bearing mice of the same strain and with two spontaneous hepatomas, one in the mouse strain of origin of the cultured mouse cells and the other in an F_1 hybrid mouse.

DISCUSSION

Several things are at once apparent from the tables. All the long-term strains of cells now have very low catalase activity. When certain of the cell strains from culture are returned to the mouse strain of origin, the tumors arising therefrom preserve the low activity of the cells from culture. Even the activity of the hepatomas growing in the animals injected with cells of the 1795 hepatoma strain is not augmented to approach that of the primary spontaneous hepatomas from the mouse. The tumors arising from reinjection of the cells into the mouse strain of origin produce a relatively small fall in the liver-catalase activity of the host mouse. It had been noted previously that tumors resulting from the injection of the cells from culture depressed the liver glycogen of the host mouse relatively little (*12*).

The catalase activity of the hepatoma strain in culture was much less than that of two spontaneous primary tumors taken directly from the mouse. There is always some question as to whether such sections taken from organs bearing tumors may have admixed normal tissue. They, of course, had some red cells, but the tumor samples appeared to be uniformly composed of tumor cells. It seems clear that the spontaneous tumors

are much higher in catalase activity than the tumors from the hepatoma strain of cells from culture. Knowledge is hardly sufficient to enable us to ascribe the decrease in catalase activity of the cultured cells to lack of oxygen in the cultures or to the malignant transformation.

TABLE 1.—Catalase activity of cells grown for long terms in culture

Strain number	Origin of the cell strain	Years in culture*	Present status of cells	Relative catalase activity, units†
721	Parenchymal epithelium of liver of young C3H/HeN mouse	10	Liver epithelium	<2
1469	One cell of 721	(10)6	Low hepatoma strain‡	<2
1795	Tumor arising from injection of 1469 cells into C3H/HeN mouse	(10)6	High hepatoma strain§	<2
929L	One cell from culture of connective tissue from flank of C3H/HeN	(19)10	Sarcoma strain	<1
2071	One culture of 929L adapted to growth in defined protein-free medium	(19)3	Sarcoma strain	<1
1742	Culture from fat pads and areolar tissue of C3H/HeN mouse; single cell from culture used	8	High sarcoma strain	<1
2049	Descended from same	8	Low sarcoma strain	<1
1745	Single cell from tumor, from 1742	(8)7	High sarcoma strain	<1
2050	Same single cell as 1745 from the tumor	(8)7	High sarcoma strain	<1
2198	Epidermis of human male, 5th decade	(5)3	Presumed to be normal epithelium	<1
1985	Human cervical carcinoma, (HeLa)	(5)3	Unknown, presumably carcinoma	<1
2435	Parotid gland of C3H/HeN mouse	1.5	Normal parotid cells, epithelium	<1

*The activities for most of these cell strains were assayed at least a year before this compilation so that, except for the parotid cells, a year should be added to the ages listed. The figures represent the average of 10 closely agreeing assays, except for strain 2435 for which 3 were made. Numbers in parentheses indicate years in culture of strain from which this strain derived.

†Relative catalase units were based on activities per unit of protein in the homogenates. We are indebted to Mr. W. R. Sterling, who worked with Dr. V. E. Price and Dr. R. E. Greenfield of the Laboratory of Biochemistry, National Cancer Institute, for 2 assays for catalase activity on strain 1469 cells, by their procedure (*11*). To approximate the absolute value of our tabulated figures their assay gave 3 units per gm. protein, whereas normal rat liver gave 900.

‡"Low hepatoma" strain means that when 142 implantations of cultured cells into the mouse strain of origin were made, 2 of the cultures gave rise to hepatomas.

§"High hepatoma strain" means that after this strain was set up from the excised hepatoma the cultures on reimplantation into the mouse strain of origin gave 100 percent tumors. However, the latest injection of 12 cultures gave no tumors (*5*).

TABLE 2.—Comparative catalase activity of various tissues from the mouse

1. Liver, normal C3H/HeN mouse	400
2. Liver, mouse bearing tumor from 1795 cells	300
3. Hepatoma recovered from injection and tumor production by 1795 cells	<2
4. Liver from mouse bearing tumor from strain 1745 cells, sarcoma	350
5. Tumor from injection of 1745 strain cells	<2
6. Liver from mouse bearing tumor from injection of strain 2050 cells	300
7. Tumor from injection of 2050 cells	<2
8. Spontaneous primary hepatoma,* C3H/HeNF mouse	150
9. Spontaneous primary hepatoma,* C3H-Vob mouse	150

*We are indebted to Mr. George Vlahakis, Laboratory of Biology, National Cancer Institute, for these tumors. For items 1 through 7, 12 separate samples were assayed. Items 8 and 9 consist of 1 assay per section on 3 sections from 1 liver (hepatoma) each. In items 3, 5, and 7, the tumors varied in size from 0.5 to 5 gm. There was little variation in activity with size of the tumor, either for the tumor or for the liver of the host.

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Susceptibility of Seven Inbred Strains and the F_1 Hybrids to Estrogen-Induced Testicular Tumors and Occurrence of Spontaneous Testicular Tumors in Strain BALB/c Mice [1]

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SUMMARY

Strain BALB/c mice are susceptible to the development of estrogen-induced interstitial-cell testicular tumors. Mice of this strain and 6 other inbred strains were tested for susceptibility to these tumors by subcutaneous implantation of pellets containing 20 percent diethylstilbestrol in cholesterol. Strain BALB/c mice (incidence = 80%) were far more susceptible than the most susceptible (strain DBA/2 = 12%) of the other strains. F_1 hybrids derived by mating BALB/c females to males of the 6 other strains were tested for susceptibility by the same procedure. Those procured from strain DBA/2 (incidence = 67%) or strain Y (incidence = 65%) were the most susceptible and those from strain I (incidence = 3%) or strain RIII (incidence = 0%) were the most resistant. The incidence of gross spontaneous testicular tumors in 154 BALB/c mice was 0 percent. Three mice had microscopic lesions of the testes. In a testis of one, connective-tissue elements predominated. Testes of the other 2 mice contained small areas of interstitial-cell hyperplasia.—J. Nat. Cancer Inst. 25: 1069–1081, 1960.

MICE OF inbred strain BALB/c have been used extensively in this laboratory for studies of estrogen-induced interstitial-cell testicular tumors. This paper reports 2 investigations in which they were used. The first was designed to compare the susceptibility of BALB/c mice to that of 6 other inbred strains to induced testicular tumors and, of more importance, to determine the susceptibility of F_1 hybrids derived by mating these strains to strain BALB/c. It was considered of interest to determine whether the high degree of susceptibility was unique for strain BALB/c and to which F_1 hybrids this predisposition was transmitted.

The second study was performed to ascertain the incidence of spontaneous testicular tumors in strain BALB/c. Previous control groups from a series of investigations, consisting of several hundred mice, had revealed no gross testicular tumors, but it was essential to determine in a more systematic manner the rate of spontaneous occurrence in this strain.

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SUSCEPTIBILITY OF INBRED MICE TO INDUCED TESTICULAR TUMORS

Bonser and Robson (*1*) and Bonser (*2*) reported the occurrence of testicular tumors in strain A mice exposed to prolonged estrogenic stimulation. Bonser (*3*) performed a well-designed experiment to ascertain the relative susceptibilities of 5 inbred strains that received subcutaneous injections of 3 mg. triphenylethylene in sesame oil. The testes were examined histologically to ascertain the degree of interstitial-cell hyperplasia and the presence of tumors. These criteria were used to establish strains A, RIII, IF, WLL, and CBA, in descending order of susceptibility.

Gardner (*4–8*) with his colleagues (*9–11*) have given some consideration to the susceptibility of inbred mice to induced testicular tumors and have published excellent reviews (*7, 8, 11*). It was found that administration of estrogens to strains A, JK, C3H, C57BL, and F_1 hybrids derived by mating strain A to strains C3H, CBA, or C57BL, and strain C57BL to strain CBA, produced testicular tumors, whereas such tumors did not occur in mice of strains $C_{12}I$, CBA, and BC, or in (BC $\times$ C57BL)F_1 hybrids. Gardner (*7, 8*) and Gardner *et al.* (*11*) have also reported that inbred mice exhibited a difference in strain response to different estrogens. For example, triphenylethylene induced testicular tumors in strain JK mice while diethylstilbestrol or estradiol benzoate did not, and C57BL mice were susceptible to tumor induction by tri-*p*-anisylchloroethylene but not by diethylstilbestrol or estradiol benzoate. Hybrids (C57BL $\times$ CBA)F_1 reacted the same as strain C57BL animals. Pertinent to the present paper was the finding that strain C3H mice were resistant to diethylstilbestrol or estradiol benzoate; of 14 receiving triphenylethylene, only 1 developed a testicular tumor which, when examined microscopically, "differed morphologically from all the others" of the experiment (*5*).

Previous experiments performed in this laboratory (*12–14*) have shown that inbred strain BALB/c mice are susceptible to the induction of testicular tumors by diethylstilbestrol, and the tumors occur after removal of the exogenous hormonal stimulation (*15*). In one study (*16*), mice of strains C3H and C57BL received diethylstilbestrol and none of the males developed a testicular tumor.

Two experiments were performed to ascertain the relative susceptibilities of inbred strains (*17*) and F_1 hybrids to the development of testicular tumors. In the first, 7 inbred strains (RIII, C57BL, C3H, DBA/2, Y, I, and BALB/c) maintained in this laboratory were used. The test mice were 3 to 6 months of age and were from colonies presumably free of the mammary-tumor agent. Diethylstilbestrol-cholesterol pellets containing 10 or 20 percent diethylstilbestrol were implanted subcutaneously into the right axilla. The 10 and 20 percent pellets were used in strains that were known to be susceptible to the toxic effects of the estrogen. All surviving mice of 3 strains (15, strain RIII; 5, strain I; 12, strain Y) were killed at 9 months and of 2 strains (29, C57BL; 13,

DBA/2) at 15 months after receiving pellets, because of their poor physical condition.

In the second experiment, F_1 hybrids derived by mating BALB/c females to males of all other strains were test animals. The mice were 2 months old when each received a 20 percent diethylstilbestrol pellet subcutaneously in the right axilla. All survivors were killed 15 to 15.5 months after implantation of pellets. The purpose of the experiment was twofold: first, to determine the susceptibility of hybrids derived from strain BALB/c to induced testicular tumors; second, to explore the possibility that they were better test animals for the study of testicular tumors than their parent BALB/c strain. Previous experience (*13*) with BALB/c had revealed that many died within a few months after receiving pellets containing diethylstilbestrol, while others developed leukosis, usually confined to the thymus gland, before they developed testicular tumors.

The results of both experiments are summarized in 2 tables; table 1 lists the occurrence of testicular tumors only and table 2 the occurrence of all tumors and scrotal hernias. Of the mice in table 1 with testicular tumors, the following developed bilateral tumors: 33 BALB/c; 2 DBA/2 that had received 20 percent diethylstilbestrol pellets; 1 (BALB/c $\times$ C57BL)F_1 hybrid; 5 (BALB/c $\times$ C3H)F_1 hybrids; 5 (BALB/c $\times$ DBA/2)F_1 hybrids.

None of the strains approached strain BALB/c in susceptibility. Strains RIII, Y, and I were sensitive to the estrogen as well as resistant to tumor development. Few tumors arose in mice of the other 3 strains. None of the (BALB/c $\times$ RIII)F_1 hybrids and only 1 of the (BALB/c $\times$ I)F_1 hybrids developed a testicular tumor. The former were most susceptible of all strains and hybrids to toxic effect of the estrogen; 9 died within 2 months after exposure.

The remaining 4 groups of hybrids showed appreciable incidences of testicular tumors. Those procured from strain C57BL males not only had the lowest incidence of 26 percent, but also developed pituitary tumors and leukoses. The next highest incidence of 44 percent occurred in hybrids derived from strain C3H males, but many developed mammary-gland tumors or scrotal hernias. Hybrids from males of strains DBA/2 and Y showed testicular-tumor incidences of 67 and 65 percent, respectively, and could serve as experimental animals. Many of those with DBA/2 fathers also developed scrotal hernias, which are an annoyance in examinations for tumors, but seem to have no influence upon their occurrence.

The results indicate that (BALB/c $\times$ Y)F_1 hybrids may be the most satisfactory substitutes for strain BALB/c mice. None developed other types of tumors, and only 2 had scrotal hernias.

Histologically the tumors in other inbred strains and in the F_1 hybrids were in every way comparable to those of strain BALB/c.

The over-all results show, however, that none of the inbred strains or F_1 hybrids were as susceptible as strain BALB/c mice, and none showed

TABLE 1.—Occurrence of interstitial-cell testicular tumors in 7 strains of inbred mice and in F_1 hybrids derived by mating BALB/c females to 6 other strains

Strain or F_1 hybrid	Diethylstilbestrol in 5 mg. pellet (*percent*)	Number of mice	Age (*months*)																					Total	Tumor incidence (*percent*)	Average age (*months*)
			2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23			
RIII	10	25		*2/0	6/0	3/0	4/0				3/0	3/0	1/0	1/0	2/0									25/0	0	—
RIII	20	20		3/0	2/0	2/0	1/0	1/0		3/0		5/0	1/0	2/0										20/0	0	—
C57BL	10	31					2/0	1/0											6/0	4/0	10/0	7/0	1/0	31/0	0	—
C57BL	20	29				1/0		1/0			4/0	2/0	2/0		2/0		4/0	2/0	6/0		1/0	3/0	0/1	28/1	3	23.0
C3H	20	32												2/0	1/1		1/0	2/0	2/1	5/0	7/0	8/0	2/0	30/2	6	17.0
DBA/2	10	25			1/0	4/0				2/0	1/0	1/0		2/0	2/1	1/0	1/1		2/0	6/0				23/2	4	16.0
DBA/2	20	25		2/0			2/0		2/0	1/0	1/0			1/0	1/1	1/1	0/1	3/0	3/0	5/0				22/3	12	16.0
Y	10	17			1/0	1/0				1/0	1/0	7/0	2/0	4/0										17/0	0	—
Y	20	14						1/0	2/0	4/0	2/0	3/0	1/0	1/0										14/0	0	—
I	20	15			1/0		2/0	1/0	2/0	2/0		1/0	6/0											15/0	0	—
BALB/c	20	51			1/0	2/0	3/0	2/3	0/6	2/11	0/8	0/9	0/3		0/1									10/41	80	10.7

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TABLE 2.—Occurrence of tumors and scrotal lesions in 7 strains of inbred mice and in F_1 hybrids after subcutaneous implantation of diethylstilbestrol-cholesterol pellets

Strain or F_1 hybrid	Diethylstilbestrol in 5 mg. pellet (*percent*)	Number of mice	Interstitial-cell testicular tumors		Mammary-gland tumors		Pituitary tumors		Leukoses*		Scrotal hernias
			Number of mice	Average age (*months*)	Number of mice	Average age (*months*)	Number of mice	Average age (*months*)	Number of mice	Average age (*months*)	Number of mice
RIII	10	25	0	—	0	—	0	—	0	—	1
RIII	20	20	0	—	0	—	0	—	0	—	1
C57BL	10	31	0	—	0	—	1	19	2	21	12
C57BL	20	29	1	23	0	—	5	17	3	16	6
C3H	20	32	2	17	4	19	1	20	1	19	1
DBA/2	10	25	2	16	0	—	0	—	0	—	15
DBA/2	20	25	3	16	0	—	0	—	0	—	16
Y	10	17	0	—	0	—	0	—	0	—	0
Y	20	14	0	—	0	—	0	—	0	—	0
I	20	15	0	—	0	—	0	—	0	—	1
BALB/c	20	51	41	11	0	—	0	—	3	9	2
BALB/c × RIII	20	37	0	—	2	13	3	14	0	—	6
BALB/c × C57BL	20	38	10	12	0	—	2	14	4	12	1
BALB/c × C3H	20	27	12	12	10	12	0	—	0	—	11
BALB/c × DBA/2	20	24	16	11	1	12	0	—	1	14	10
BALB/c × Y	20	26	17	11	0	—	0	—	0	—	2
BALB/c × I	20	37	1	11	0	—	0	—	7	11	8
BALB/c	20	25	20	8	0	—	0	—	4	8	2

*Including generalized lesions and those confined to the thymus gland.

sufficient advantages to displace BALB/c as the strain of choice for studies of induced testicular tumors.

Too many factors, including the use of different estrogens, techniques of administration, and substrains of mice, were involved to attempt a serious comparison of the present findings with those of other investigators. Among the strains used here only 2 were used by others to compare the susceptibilities of inbred mice. The resistance of strain C3H confirmed Gardner's (*8*) findings, but the resistance of strain RIII differed from Bonser's (*3*) results. She observed the occurrence of testicular tumors in 14 of 38 RIII mice that survived treatment for 50 weeks or more. The difference between her findings and those reported here could be attributed to substrain susceptibility and the use of different estrogens. Her mice received triphenylethylene in sesame oil, while ours received pellets consisting of diethylstilbestrol dissolved in cholesterol. Also, only 15 of our mice survived for more than 50 weeks.

There is general agreement that the inbred strains BALB/c and Strong's line of strain A are relatively susceptible to estrogen-induced interstitial-cell testicular tumors, and it is of interest that these strains are related (*17*, *18*). Both are also susceptible to the occurrence of spontaneous and induced pulmonary tumors (*19*) as well as to the development of induced hepatomas (*20*). Likewise, while carrying the mammary-tumor agent, both strains show a high incidence of mammary tumors, provided they bear litters (*19*, *21*). Among the inbred strains resistant to induced testicular tumors, strains C3H, CBA, and $C_{12}I$ have a common ancestry (*22*).

The response of F_1 hybrids derived from BALB/c females and males of the other 6 strains provided an excellent example of the wide variations in susceptibility displayed by outcross animals. The incidences of testicular tumors in these mice ranged between 0 and 67 percent. Those derived from RIII fathers (incidence = 0%) showed an incidence of 0 percent, and those from I fathers (incidence = 0%) an incidence of 3 percent. In contrast, those procured from strain DBA/2 fathers (incidence = 4%) exhibited an incidence of 67 percent and those from Y fathers (incidence = 0%) an incidence of 65 percent. Those from C3H (incidence = 6%) and C57BL (incidence = 3%) fathers were of intermediate susceptibility and showed incidences of 44 and 26 percent respectively. None of the (BALB/c × Y)F_1 hybrids developed bilateral testicular tumors.

The occurrence of mammary tumors, pituitary tumors, and leukoses in the intact males of these experiments was compared with the same type of tumors appearing in castrated males (*23*) that received diethylstilbestrol pellets. The present results confirmed the earlier findings as far as mammary and pituitary tumors were concerned but, in general, more castrates developed leukoses. This difference was most pronounced in the (BALB/c × I)F_1 hybrids; leukosis occurred in 17 of 45 castrates and in 7 of 37 intact males.

SPONTANEOUS TESTICULAR TUMORS IN BALB/c MICE

There are several papers by two groups of investigators in which they reported the occurrence of spontaneous testicular tumors in mice. The first group, Slye, Holmes, and Wells (*24*), recorded such tumors in 28 of 9,500 male mice of the Slye stock which were 9 to 42 months old. Tumor size varied between 5 mm. in diameter to 30 × 23 × 23 mm. Two were diagnosed as spindle-cell sarcomas, one represented a tumor of interstitial cells, but most consisted of "cells closely resembling the epithelium of the seminiferous tubules, arranged in an alveolar structure." Twenty-seven of the tumors arose in mice of strain 90 and in their hybrids, but the tumor incidence of the strain was not mentioned.

Of the second group, a paper by Athias (*25*) described the establishment of strain H in which the males developed spontaneous mammary and testicular tumors. Furtado Dias (*26*) has supplied recent information concerning this strain, and her latest publication (*27*) contained a summation of the findings. Strain H was established in 1938 and shows an incidence of mammary tumors of up to 66 percent in females. When about 18 months of age, males also develop mammary tumors. Of importance here is the occurrence of testicular lesions in almost all males with or without mammary tumors. The lesions are always bilateral and sufficiently severe to make difficult the maintenance of the strain. In mice 3 to 4 months old, changes "may already be observed in the seminiferous tubules, accompanied by a greater or lesser degree of hypertrophy of the Leydig cells." In some instances the hypertrophy is pronounced and, to date, "some twenty" mice have presented interstitial-cell tumors. These papers do not include the size and incidence of the tumors or the age of the mice in which they were found.

Various investigators have reported the occurrence of sporadic testicular tumors in inbred mice (*17*). Bittner (*28*) found an embryonal-cell carcinoma of the testis in an 11-month-old strain I mouse. Gardner (*29*) found 3 testicular tumors in 61 reciprocal F_1 hybrids derived by mating strain A to strains C3H or C57BL. An interstitial-cell tumor arose in a (C3H × A)F_1 hybrid that was autopsied when 31.5 months old. Two tumors were in (C57BL × A)F_1 hybrids autopsied when 27 months old; one was an interstitial-cell tumor, and the other "probably an interstitial-cell tumor but resembled a seminoma rather than the usual interstitial-cell tumor." Hooker, Strong, and Pfeiffer (*30*) reported an interstitial-cell tumor in an untreated mouse of the Strong C strain, which was transplanted successfully into untreated hosts of the same strain.

Clifton *et al.* (*31*) found an interstitial-cell tumor of the testis in a 16-month-old mouse of the RF strain and carried it through 10 transfer generations in RF mice. The authors stated, "The rate of occurrence of such tumors in this strain is less than 1 percent." During recent years Stevens (*32*) carried out an interesting series of investigations with strain 129 mice. About 1 percent develop congenital testicular teratomas composed of numerous types of tissue.

The only record found of a spontaneous testicular tumor in a BALB/c mouse was by Hummel (*33*) who detected in an 18-month-old animal an interstitial-cell tumor that grew when transplanted into BALB/c mice of both sexes. Huseby and Bittner (*34*) and Huseby (*35*) reported the occurrence of interstitial-cell tumors in untreated cryptorchid BALB/c males. In 15 mice, 1 month old, the testes were placed well up in the abdominal cavity and, after an average of 22.9 months, all testes, though of small size, contained areas of interstitial-cell hyperplasia, 7 of which were classified as interstitial-cell tumors. In 16 cryptorchid mice whose testes remained near the inguinal ring for an average time of 22.6 months, 5 testes showed significant areas of hyperplasia and of these only 2 contained typical tumors. In another experiment Huseby (*35*) implanted minced testes from 12 BALB/c mice into the subcutaneous tissues of 60 other castrate BALB/c males that received 10 percent diethylstilbestrol-cholesterol pellets, and interstitial-cell tumors arose in 10 of the recipient mice at an average of 14.5 months after implantation of the testicular mince. Apparently the abdominal cavity provided an environment in which these tumors developed in BALB/c mice that were not exposed to exogenous estrogens.

To ascertain the incidence of spontaneous testicular tumors in BALB/c males a total of 154 mice were used. They were examined at biweekly intervals for the presence of enlarged testes. Seventeen died before the age of 1 year, with 137 mice left and, of these, 59 died or were killed when 13 to 23 months old, at an average age of 20 months. None developed enlarged testes and all testes were grossly normal at the time of death. The surviving 78 mice were killed when 24 to 25 months old. Twenty-three were free of gross tumors, and 9 each had 2 types of tumor. Forty-five developed pulmonary tumors, 10 developed leukoses, 7 developed hepatomas, 1 had a hemangioma, and 1 a reticulum-cell sarcoma.

Three mice developed testicular lesions. In one (#11), when 22.5 months old, the right testis was 9 mm. long. It remained at about the same size for 2 months and measured $8 \times 6 \times 5$ mm. when the mouse was killed. The other testis was of normal size and appearance. When examined microscopically, the enlarged testis was a mass of connective tissue with a semblance of testicular tubules. Some blood-vessel spaces were surrounded by thick scars. A few blood-pigment-laden macrophages were visible. The picture could have been the end result of an interstitial-cell tumor, but no elements remained to make the diagnosis even after semiserial section of the remaining specimen.

Both other testicular lesions were found at autopsy in 2-year-old mice. The first (#52) had small brown testes with thickened capsules containing a few flecks of calcium and consisting of elements of interstitial cells and some faint evidence of tubules. In contrast with the usual hormone-induced tumors, the tubular remnants were not displaced to one side, but appeared to be scattered throughout the interstitial-cell tissue. There were many large cells containing blood pigment, a typical feature of old testicular tumors. The entire mass was small, and the cellular

process appeared to be indolent, but definitely represented a small tumor of interstitial-cell type (figs. 1 and 2).

The second mouse (#80) had testes of normal size, but the left testis contained small, yellow spots which were grossly visible. Histologically the testis was atrophic, with no evidence of spermatogenesis. In one subcapsular area was a small nodule of interstitial cells which blended diffusely with adjoining interstitial spaces. It was a definite area of interstitial-cell hyperplasia (figs. 3 and 4).

Thus no gross tumors were observed, if the previous definition of "tumor" as a neoplastic enlargement of the testes to 9 mm. or more in length is maintained (*14*). The only testis reaching that size could not be diagnosed as a tumor, and the other two lesions occurred in testes that were either normal or smaller than normal in size. The evidence indicates that interstitial-cell hyperplasias occur spontaneously in BALB/c mice, but the frequency is low, probably under 1 percent, in animals living 12 to 24 months.

CONCLUSIONS

Strain BALB/c mice were far more susceptible to estrogen-induced interstitial-cell testicular tumors than strains RIII, C57BL, C3H, DBA/2, Y, or I. Of F_1 hybrids derived by mating BALB/c females to males of the other 6 strains, those with DBA/2 or Y fathers were the most susceptible, those with C3H or C57BL fathers were of intermediate susceptibility, and those with I or RIII fathers were the most resistant. The incidence of tumors in F_1 hybrids ranged between 0 percent in (BALB/c $\times$ RIII)F_1 hybrids and 67 percent in (BALB/c $\times$ DBA/2)F_1 hybrids. None of the hybrids were as susceptible as the parent strain BALB/c.

The incidence of spontaneous tumors in untreated BALB/c mice was less than 1 percent.

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PLATE

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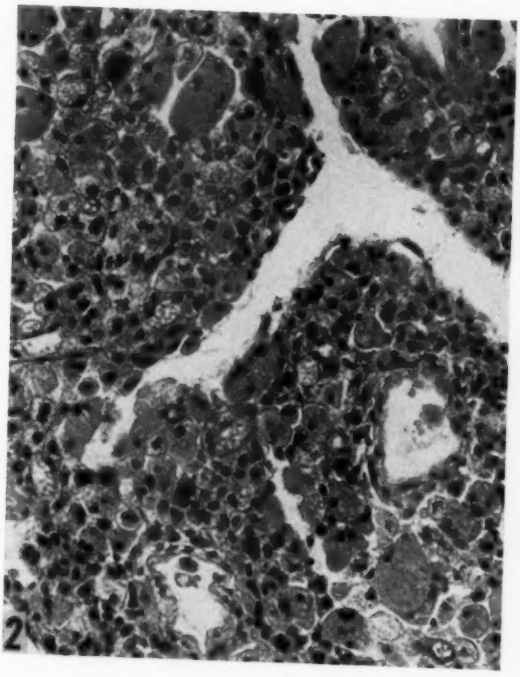
PLATE 147

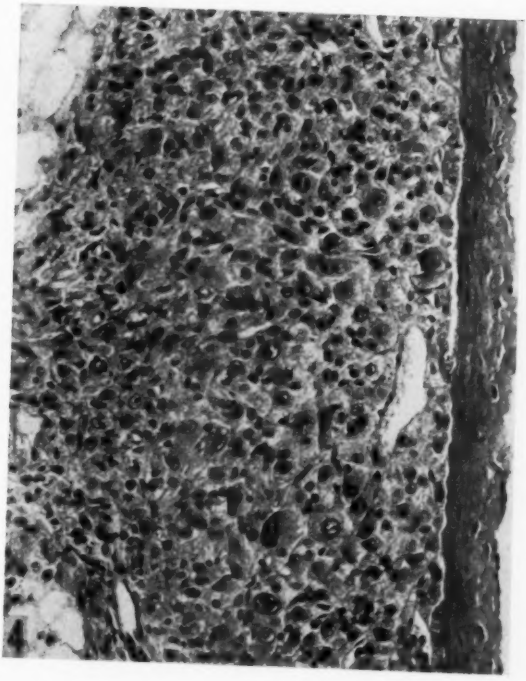
FIGURE 1.—Testis of BALB/c mouse (#52), 24 months old, consisting of interstitial cells with few fragments of tubular elements. Hematoxylin and eosin. × 15

FIGURE 2.—Higher magnification of figure 1 showing interstitial-cell tumor. × 200

FIGURE 3.—Testis of BALB/c mouse (#80), 24 months old, with subcapsular area of interstitial hyperplasia. No active spermatogenesis was identified in tubular elements. Hematoxylin and eosin. × 20

FIGURE 4.—Higher magnification of figure 3, showing interstitial-cell nodule. × 200





Some Factors Involved in the Induction or Growth of Testicular Tumors in BALB/c Mice [1]

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SUMMARY

The effects of interruption of estrogen after second exposure to estrogenic stimulation, age of the animals, and treatment with progesterone were studied on the development and growth of interstitial-cell tumors of the testes in BALB/c mice implanted subcutaneously with diethylstilbestrol-cholesterol pellets. (1) Estrogenic stimulation for 8 weeks, removal of the pellet for 24 weeks, and reimplantation of a second pellet delayed the occurrence of tumors by approximately the intervening nonestrogen period, which indicated that the process was interrupted, but did not progress or regress to any appreciable extent during that period. (2) Diethylstilbestrol pellets implanted in mice from 2 weeks to 12 months of age showed that all age groups responded with the appearance of testicular tumors. The tumors appeared later and reached a lower cumulative frequency in the 2-week-old mice, and a smaller proportion grew progressively after removal of diethylstilbestrol from the 12-month-old mice. (3) Progesterone retarded but did not prevent the occurrence of estrogen-induced tumors under the conditions of the experiment.—J. Nat. Cancer Inst. 25: 1083–1096, 1960.

IN A previous paper (*1*) dealing with the growth of estrogen-induced interstitial-cell testicular tumors in BALB/c mice, attention was directed to the wide variation in growth patterns of the tumors as evidence that they represent a biologically unstable system. This implies that a number of systemic and local factors are involved in their occurrence and growth. Further investigations of possible factors that may modify this type of carcinogenesis thus appeared indicated.

The present paper reports efforts to ascertain the effects of interruption of estrogenic stimulation, after a second exposure, the age of the host, and progesterone on the occurrence or growth of the induced tumors. In all three experiments, the definitions and conditions described previously (*1, 2*) were followed systematically. It is important to recapitulate that the term "tumor" was applied to testes that reached a minimum of 9 to 10 mm. long. Histologic examination showed that the tumors were uniformly of the interstitial-cell type.

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[2] We are indebted to Miss Joan M. Gurian for statistical assistance.
[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

RESPONSE TO A SECOND ESTROGENIC STIMULUS

During earlier investigations 2-month-old BALB/c males received diethylstilbestrol-cholesterol pellets containing 20 percent diethylstilbestrol that were removed 4 to 28 weeks later when the testes were of normal size and appearance (*2*). The mice received second 20 percent diethylstilbestrol pellets 5 to 12 months after removal of the first pellets, and 34 percent developed testicular tumors within 4 months. Tumors arose in 50 percent of mice that had carried their first pellets for 24 or 28 weeks and in 12 percent of those exposed for 16 weeks. The findings were interpreted as showing that the earlier period of hormonal stimulation had conditioned the testes to react promptly to a second stimulus.

The results in mice that had carried pellets for 24 or 28 weeks were clear, but it was deemed advisable to investigate further the response of those exposed to the first stimulus for shorter periods by using a uniform procedure and including appropriate controls.

All pellets were inserted subcutaneously, by trocar, into the right axilla. Each pellet weighed about 5 mg. and was prepared by fusing 20 parts of diethylstilbestrol with 80 parts of cholesterol.

The experimental mice, groups 8E and 16E, were 2 months old when they received first pellets that were excised 8 or 16 weeks later, respectively. Second pellets were implanted 24 weeks after removal of the first. Two groups of controls were used for each experimental group. The first, groups 8EC and 16EC, received pellets when 2 months of age, carried pellets for 8 or 16 weeks, and were not exposed to second pellets. The results in these mice were published elsewhere (*3*). The second, groups 8C and 16C, received pellets when the experimental mice received their second pellets, or when 9 and 11 months old, respectively.

The results are summarized in table 1. Mice from experiment 300A of another study (*1*) are included in the table for comparison. They received 20 percent diethylstilbestrol pellets when 2 months old and retained the pellets until the occurrence of tumors. With the exception of groups 8EC and 16EC that carried pellets for only 8 and 16 weeks, respectively, all groups showed similar incidences of tumors.

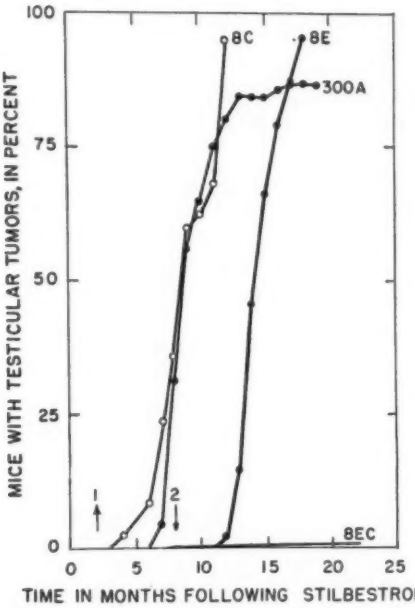
The data of table 1 are presented graphically in text-figures 1 and 2, in which the cumulative frequencies have been calculated by the actuarial method. The figures demonstrate that the removal of the hormonal stimulus for 24 weeks merely displaced the time of tumor appearance by approximately this same period. This would indicate that the process of tumor induction and growth was terminated by removing the diethylstilbestrol pellet after 8 weeks and was resumed by the reintroduction of the second pellet. During the interval of 24 weeks, apparently there was no regression or progression of the neoplastic process, which resumed promptly as soon as the hormonal stimulus was reintroduced (text-fig. 1). However, when the first pellet remained in the animal for 16 weeks, approximately 25 percent of the tumors continued to grow progressively and became manifested as gross tumors. Progressive growth of all the

TABLE 1.—Occurrence of testicular tumors in BALB/c mice after second exposure to 20 percent diethylstilbestrol pellets and in control mice

Group*	Number of mice	Age (*months*)																							Total	Tumor incidence (*percent*)	Average age (*months*)	Average age at death without tumors (*months*)
		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24				
8EC	48	2†/0	1/0						1/0	3/0	1/0	2/0	1/0	2/0	2/0	2/0	3/0		5/0	2/0	8/0	1/0	2/0	10/0	48/0	0	—	17.6
8E	57	3/0	1/0			1/0			8/0	1/0	1/1	1/5	0/13	1/8	0/5	0/3	1/3	1/0							19/38	67	13.8	8.9
8C	53	1/0	1/0	1/0		1/0			4/0		0/1	1/2	2/1	1/6	1/5	1/9	1/1	0/2	3/8						18/35	66	15.9	11.6
16EC	124	3/0	2/0	3/0	1/0	2/0	1/0	2/1	5/0	2/1	8/1	2/1	1/0	2/2	3/3	4/0	2/1	6/4	3/0	4/0	10/3	4/1	6/0	30/0	106/18	15	16.0	17.2
16E	79	4/0	4/0	1/0							15/1	2/7	0/6	1/6	1/11	0/8	2/5	0/3	1/0	1/0					32/47	59	14.7	9.8
16C	79				1/0		1/0		1/0	1/0	3/0	3/0	1/0	1/0	2/5	3/7	3/17	2/14	1/4	0/1	0/2	0/3	1/0	2/0	26/53	67	17.6	14.7
E300A	101	2/0	1/0	2/0	1/0	4/4	5/24	1/21	0/7	2/8	0/4	0/3			0/1	0/1	4/0	6/0							28/73	73	8.4	10.5

*8EC—Carried pellets for 8 weeks.
8E—Carried pellets for 8 weeks; pellets out for 24 weeks; received second pellets.
8C—Received pellets when group 8E received second pellets.
16EC—Carried pellets for 16 weeks.
16E—Carried pellets for 16 weeks; pellets out for 24 weeks; received second pellets.
16C—Received pellets when group 16E received second pellets.
E300A—Carried pellets from 2 months of age (*1*).

† Number dead without tumors / Number with tumors.



TEXT-FIGURE 1.—Cumulative frequency of testicular tumors in BALB/c mice implanted with 20 percent diethylstilbestrol pellets for different periods.

8E—Mice received pellets at 2 months of age. Pellets were removed after 8 weeks (at *arrow* 1) and mice received another pellet 24 weeks later (at *arrow* 2).

8EC—Two-month-old mice received pellets which were removed after 8 weeks (at *arrow* 1).

8C—Mice received pellets when 9 months old, or when 8E mice received second pellets.

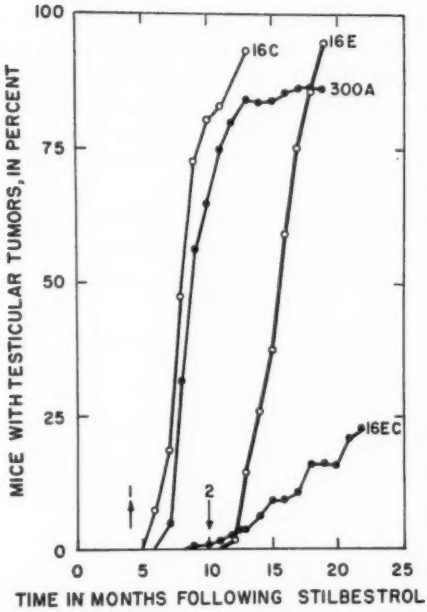
300A—Previous series of 2-month-old mice bearing diethylstilbestrol pellets until tumors appeared, which showed similarity to 8C.

tumors was also achieved by reintroduction of the second pellet (text-fig. 2).

The results are also interpreted as showing that exposure to the estrogen for 8 weeks produced interstitial-cell changes that persisted for at least 24 weeks and reacted promptly to a second stimulus. Histologic examination of testes did indeed show small nodules of interstitial hyperplasia. Thus, 8 weeks' exposure to 20 percent diethylstilbestrol pellets was above the amount of hormone necessary to initiate the irreversible response. Exploration of lower-dose ranges would therefore be of interest and is receiving consideration.

AGE FACTOR IN SUSCEPTIBILITY TO INDUCED TESTICULAR TUMORS

A review of the literature revealed that the majority of investigators interested in the production of interstitial-cell testicular tumors in mice



TEXT-FIGURE 2.—Cumulative frequency of testicular tumors in BALB/c mice implanted with 20 percent diethylstilbestrol pellets for different periods.

16E—Mice received pellets at 2 months of age. Pellets were removed after 16 weeks (at *arrow* 1) and mice received another pellet 24 weeks later (at *arrow* 2).

16EC—Two-month-old mice received pellets that were removed after 16 weeks (at *arrow* 1).

16C—Mice received pellets when 11 months old, or when 16E mice received second pellets.

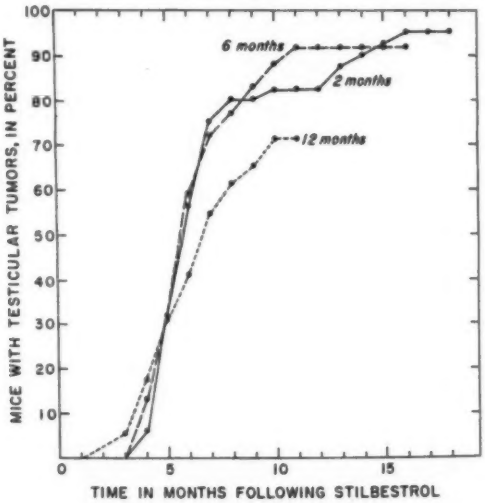
300A—Previous series, and same as in text-figure 1; 2-month-old mice bearing diethylstilbestrol pellets until tumors appeared, which showed similarity to 16C.

began administering estrogens to mice that were 4 to 6 weeks of age. Shimkin, Grady, and Andervont (*4*) used mice "up to 3 months of age"; Shimkin and Andervont (*5*) started with mice 1.5 to 3 months of age, and Gardner (*6*) stated that his animals were 33 to 91 days old. A reference to the possible influence of age upon susceptibility to the development of testicular tumors was not found.

The preceding experiment indicated that mice 9 and 11 months old were equally susceptible to the induction of testicular tumors as mice that were 2 months of age at the implantation of diethylstilbestrol. These observations were more systematically studied as follows.

Two experiments were performed in which mice of different ages were used. In the first, BALB/c mice 2, 6, and 12 months old received diethylstilbestrol-cholesterol pellets subcutaneously in the right axilla. Each pellet weighed about 5 mg. and contained 20 percent diethylstilbestrol. The mice were examined at biweekly intervals for the occurrence of tumors, and the criterion for the presence of a gross tumor was when one or both testes reached 9 to 10 mm. in length.

The over-all results are shown in table 2. Text-figure 3 presents the cumulative frequencies determined by the actuarial method and shows that approximately 90 percent of the 2- and 6-month-old mice developed tumors, whereas approximately 70 percent of the 12-month-old animals developed tumors. The difference between the 2- and the 12-month groups, 11 months after receiving pellets, is not significant statistically.



TEXT-FIGURE 3.—Cumulative frequency of testicular tumors in BALB/c mice after subcutaneous implantation of 20 percent diethylstilbestrol-cholesterol pellets at the age of 2, 6, or 12 months.

After a testis measured 9 to 10 mm. in length, the mouse was examined each week, and when the testis reached 11 to 12 mm. long, the pellet was removed. This was done because it was known that after the removal of pellets some testes continued to enlarge, some persisted at about the same size until the death of the animals, and some regressed to normal size; of those that regressed, some recurred while others did not before death of the host (*1*, *2*). It was hoped that these responses would assist in evaluating the effect of age upon the fate of the tumors after removal of the hormonal stimulus.

Table 3 shows the length of the testes when the pellets were excised and the time the pellets remained in the mice after they had developed tumors. The lower size range of 9 mm. and time range of 0 days were due mostly to the appearance of a second tumor in mice when the first tumor was 11 to 12 mm. long. The testes of the 12-month-old mice required, on the average, about twice the time to become at least 11 mm. long as did the testes in the other age groups.

Table 4 shows the fate of tumors after removal of pellets; 1 mouse of the 2-month-old group, 3 of the 6-month-old group, and 16 of the 12-

TABLE 2.—Occurrence of testicular tumors in BALB/c mice of different ages after receiving 20 percent diethylstilbestrol-cholesterol pellets subcutaneously

Age of mice (*months*)	Number of mice	Age (*months*)																				Total	Tumor incidence (*percent*)	Average age (*months*)	Average age at death without tumors (*months*)
		2	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23				
2	55	3*/0	2/0	2/3	2/12(6)	1/11(4)	1/8(3)	0/2		0/1			0/2	0/1	0/1	0/1		2/0				13/42(13)	76	8.9	7.6
6	74			6/0	6/0	1/0		0/8(2)	0/11(8)	0/17(6)	0/8(4)	0/3	0/4(2)	0/3	0/2(1)			2/0	1/0	2/0		18/56(23)	76	12.3	10.6
12	79									7/0	3/0	0/2	0/2(2)	1/8(4)	0/9(8)	2/7(5)	4/8(3)	1/4(1)	1/2	14/2	2†/0	35/44(23)	56	18.0	18.4

*Number dead without tumors / Number with tumors; numbers in parentheses are mice with bilateral tumors

†Survivors killed when 22 and 23 months old.

TABLE 3.—Size of testes when pellets were removed and time between the occurrence of tumors and removal of pellets

Age of mice (*months*)	Length of testes when pellets were removed (*mm.*)		Time pellet removed after tumor arose (*days*)	
	Average	Range	Average	Range
2	10.4	9–12	11.5	0–50
6	10.1	9–12	12.0	0–56
12	9.8	9–11	24.4	0–72

month-old group died before their pellets were excised. Data in the table include only those tumors appearing within 10 months after insertion of pellets because surviving mice of the 12-month-old group were killed when 22 and 23 months of age.

TABLE 4.—Fate of tumors after removal of diethylstilbestrol pellets

Age of mice (*months*)	Number of tumors	Fate of tumors							
		Grew progressively		Persisted to death of host		Regressed and no recurrence		Regressed and recurred	
		Number	Percent	Number	Percent	Number	Percent	Number	Percent
2	48	9	18	7	15	24	50	8	17
6	73	16	22	15	21	33	45	9	12
12	49	1	2	21	43	25	51	2	4

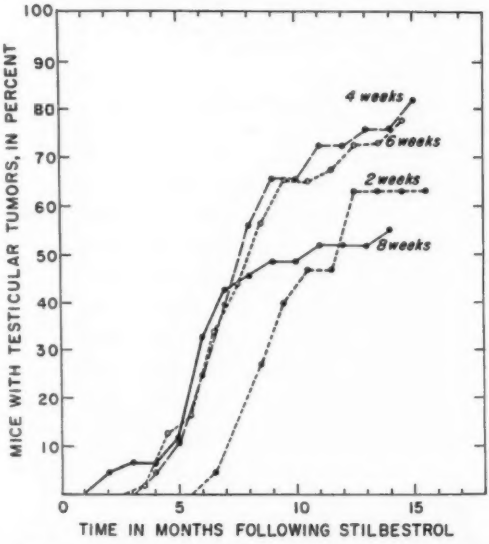
Only 2 percent of the tumors in the 12-month-old mice grew progressively, in contrast to 18 percent in the 2-month-old group and 22 percent in the 6-month-old group. The differences between the 12-month-old animals and the 2 younger groups are statistically significant ($P < 0.01$). Also, 43 percent in the oldest group persisted in contrast to 15 and 21 percent in the others. The differences between the 12-month-old mice and the 2 younger groups are statistically significant ($P < 0.02$). This is further evidence that the old animals were more resistant to the continued progressive growth of the tumors. However, the percent of tumors regressing in the 3 groups is essentially the same.

The measurements of gross tumors, indicating slower and less progressive growth in the oldest animals, were in good agreement with histologic observations. Many tumors in the 12-month-old group showed striking morphologic evidence of retrogressive changes, such as fibrosis, calcification, and relatively small areas of tumor cells that appeared to be invasive.

The second experiment was designed to ascertain the susceptibility of mice less than 2 months of age. BALB/c males 2, 4, 6, or 8 weeks old received, subcutaneously in the right axilla, diethystilbestrol-cholesterol

pellets containing 10 percent diethylstilbestrol. The smaller amount of diethylstilbestrol was used because it was known that in the younger animals a higher dose of estrogen would be lethal.

The findings are shown in table 5 and text-figure 4. In the table, the low proportion of 2-week-old mice developing tumors is due to the high mortality before the appearance of tumors, undoubtedly attributable to diethylstilbestrol. When the non-tumor mortality is taken into account by actuarial methods, as in text-figure 4, it is seen that eventually over 60 percent of the 2-week-old mice developed tumors. The differences between the 4 groups are not striking, though there is a suggestion that the tumors appeared somewhat later and reached a lower cumulative frequency among 2-week-old mice than the 4- and 6-week-old animals. The explanation for the change in slope in the 8-week-old group is obscure at this time.



TEXT-FIGURE 4.—Cumulative frequency of testicular tumors in BALB/c mice after subcutaneous implantation of 10 percent diethylstilbestrol-cholesterol pellets at the age of 2, 4, 6, or 8 weeks.

Histologically the testicular tumors were identical with those described previously (*2*); they were interstitial-cell masses with frequent cystic and hemorrhagic areas. Normal testes of 2- and 4-week-old mice were immature, grossly and histologically. At 2 weeks the cross-sectional area of the testes ranged between 1 to 1.5 × 2 to 3 mm. and at 4 weeks 2 to 4 × 4 to 5 mm. At 2 weeks the seminiferous tubules consisted of spermatocytes, with relatively few spermatogonia and no spermatozoa. At 4 weeks spermatogonia predominated and rare spermatozoa-like forms were also present. Interstitial-cell islets were present at both ages but were more prominent at 4 weeks.

TABLE 5.—Occurrence of testicular tumors in BALB/c mice of different ages after receiving 10 percent diethylstilbestrol-cholesterol pellets subcutaneously

Age of mice (*weeks*)	Number of mice	Age (*months*)																Totals	Tumor incidence (*percent*)	Average age (*months*)	Average age at death without tumors (*months*)
		0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16*				
2	61	3/0†	2/0	0/0	0/0	5/0	6/0	4/0	3/1	3/2	0/2	1/2	0/1	1/0	1/2		4/2	51/10	16	9.8	5.3
4	64			1/0	3/0	3/0	2/2	2/4	3/7	4/7	0/7	1/4	4/0	0/2		0/1	6/1	29/35	53	8.5	8.6
6	59			1/0	2/0	1/0	0/1	1/6	1/2	1/9	1/5	2/6	1/4		1/1	1/2	0/1	22/37	63	9.0	11.1
8	50			2/0		3/2	0/1	3/0	0/2	0/9	2/4	2/1	0/1	2/0	0/1	1/0	13/1	28/22	44	8.4	11.4

*Survivors killed at 16 months of age.

†Number dead without tumor / Number with tumors.

Six normal-sized testes from the 2-week-old mice that had developed unilateral tumors were examined microscopically. Of 2 from 8-month-old mice, 1 was fairly normal and 1 contained 2 small areas of interstitial-cell nodular hyperplasia. Of 2 from 9-month-old mice, 1 was fairly normal with slight interstitial-cell hyperplasia, and 1 contained 2 small nodules of hyperplasia. One from a 10-month-old animal was atrophic, but normal otherwise, and 1 from a 13-month-old mouse was normal. In addition, both normal-sized testes were examined from 2 mice of the 2-week-old group that had carried pellets 5 and 8 months, respectively. In the first, 1 testis was atrophic only and the other showed a slight degree of diffuse hyperplasia; in the second, both normal-sized testes were normal histologically. Apparently some testes of the 2-week-old mice continued their normal or near normal development in the presence of the estrogen.

Further work is needed to ascertain the relative susceptibilities of 2- and 8-week-old mice to the occurrence of testicular tumors and to the tendency of the tumors to grow progressively after removal of hormonal stimulation. Perhaps the use of 5 percent diethylstilbestrol pellets would be useful; in an earlier experiment (*2*) testicular tumors arose in only 15 percent of 2-month-old BALB/c mice after they had received such pellets.

The experiments indicate that 2-, 6-, or 12-month-old BALB/c males did not differ significantly in susceptibility to the induction of gross interstitial-cell testicular tumors, but the 12-month-old mice were more resistant than the younger groups to the progressive growth of the tumors both before and after the hormonal stimulus was removed.

When 2-, 4-, 6-, or 8-week-old BALB/c mice were exposed to the same degree of estrogenic stimulation by diethylstilbestrol and the high non-tumor mortality of the 2-week-old animals was taken into account, there was no striking difference in their degrees of susceptibility to the occurrence of induced testicular tumors. However, this problem deserves further consideration.

EFFECT OF PROGESTERONE UPON THE OCCURRENCE OF INDUCED TESTICULAR TUMORS

Few investigators have attempted to prevent the development of interstitial-cell testicular tumors in mice. Hooker and Pfeiffer (*7*) administered estradiol benzoate to one group of strain A mice and to another a mixture of estradiol benzoate and testosterone propionate. They concluded, ". . . the androgen appeared to retard, but not to prevent, the changes in the testis." According to Gardner, Pfeiffer, and Trentin (*8*), Trentin found that progesterone retarded the formation of estrogen-induced testicular tumors. Ely (*9*) used strain A mice to show that an antigonadotrophic serum inhibited the intensive interstitial-cell changes produced by estrogen stimulation.

Blivaiss, Hanson, Rosenzweig, and Kutuzov (*10*), in an abstract, recorded some evidence of inhibition. Three groups of 4- to 5-week-old strain A mice were used. The first received weekly injections of diethylstilbestrol; the second received a combination of diethylstilbestrol and progesterone; the third received progesterone. Testicular tumors appeared in 76 percent of the first group of 55 and in 51 percent of the second group of 45, a difference that is statistically significant. The average age at which tumors occurred was not stated, but the first tumor in diethylstilbestrol-treated mice was in a 4-month-old animal and, of 42 mice with tumors, 37 had bilateral tumors. In mice receiving the combined treatment, the first tumor was in a 7-month-old animal and, of 23 with tumors, 14 had bilateral tumors.

One experiment was performed in which BALB/c mice were used to ascertain the effect of progesterone upon the occurrence of testicular tumors induced by diethylstilbestrol. The mice were 2 to 3 months old when each received a diethylstilbestrol-cholesterol pellet subcutaneously in the right axilla. Each pellet weighed about 5 mg. and contained 20 percent diethylstilbestrol. One week later the administration of progesterone was started. Pellets of solid progesterone, each weighing about 15 mg., were inserted subcutaneously in the interscapular region. The treatment was continued at intervals of 3 weeks until each mouse had received 12 progesterone pellets. The aim was to maintain continuous exposure to the steroid. Previous experience with strain C3H mice had shown that a 15-mg. pellet was usually absorbed within 4 to 6 weeks when inserted subcutaneously.

The mice were examined at biweekly intervals for the presence of tumors, namely, when one or both testes measured 9 to 10 mm. long. The progesterone-treated group showed a lower incidence of tumors and a higher average age for the occurrence of tumors (table 6). The average age of 11.6 months for the occurrence of tumors is the highest encountered thus far in this laboratory when 2-month-old BALB/c males were exposed to 20 percent diethylstilbestrol pellets and permitted to live their normal lifespans. However, the difference between the diethylstilbestrol group and the diethylstilbestrol plus progesterone group is not statistically significant.

The only permissible conclusion is that the occurrence of tumors was somewhat retarded but not prevented in the progesterone-treated mice. A lower dose of diethylstilbestrol and higher dose of progesterone may give more definite results. Also, the use of progesterone in different laboratories has yielded conflicting results when tested for its influence upon the occurrence of breast tumors (*11*). Apparently dosage, time of administration, and host factors play important roles.

CONCLUSIONS

The remarkable biologic individuality of hormone-induced testicular tumors in BALB/c mice, including even bilateral tumors, is evidence that

TABLE 6.—Effect of progesterone upon the occurrence of diethylstilbestrol-induced testicular tumors in strain BALB/c mice

Treatment	Age of mice (*months*)	Number of mice	Age (*months*)																	Totals	Tumor incidence (*percent*)	Average age (*months*)	Average age at death without tumor (*months*)
			3	4	5	7	8	9	10	11	12	13	14	15	17	18	19	20	21				
S*	2–3	36	2/0†			1/1	0/7	0/9	2/6	0/2	0/2				0/1	0/1			2/0	7/29	81	9.8	10.7
S + P	2–3	36		1/0	1/0		1/2	0/5	0/3	1/3	1/1	0/6	1/1	0/1	1/0	0/2	1/0	1/0	3/0	12/24	67	11.6	14.2

*S = Diethylstilbestrol; P = progesterone.

†$\frac{\text{Number dead without tumors}}{\text{Number with tumors}}$.

many factors, both general and local, are implicated in their occurrence and growth. The observations reported here indicate that previous exposure to hormonal stimulation and the age of the host are two such factors. The fact that progesterone exerted a mild inhibitory influence upon the time of occurrence of the tumors suggests that other hormones, or combinations of hormones, other than estrogens, are involved.

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Effects of Cortisone in Tumor Transplantation [1,2]

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SUMMARY

In these studies, the observations of earlier workers that pretreatment with cortisone effectively aids in the growth of transplanted Sarcoma 180, Krebs-2, and Ehrlich ascites tumor cells have been confirmed. In a series of experiments, we made the following observations: If a minimally effective dose of cortisone was used, it had to be given before cell inoculation. With the use of cortisone, single Ehrlich cells could be consistently grown in subcutaneous tissues. Pretreatment with cortisone reduced the number of intravenously injected tumor cells required for the establishment of comparable numbers of tumor implants from approximately 2 million to only 5,000. The predominant site of metastases shifted from the lung in normal mice to the liver in cortisone-treated animals.—J. Nat. Cancer Inst. 25: 1097–1110, 1960.

PROBABLY HOWES (*1*) deserves credit for the first publication in which cortisone was described as an active agent affecting tumor transplantation. Foley and Silverstein (*2*) reported in 1951 that cortisone had a chemotherapeutic effect on C3H lymphosarcoma in CF_1 mice. Others also found inhibition of the growth of various transplanted mouse tumors. The same year, Foley further reported that strain-specific tumors could be grown in other mouse lines if the animals were pretreated with cortisone.

The unexpected observations reported by Agosin *et al.* (*3*) in 1952, that mice treated with cortisone developed many metastases even though growth of the mother tumor was arrested, stimulated many subsequent, frequently conflicting, reports. A breast tumor which had been passed for 51 generations was used in the same strain (C3H) in which the tumor had originated, and the mice were given daily injections of 0.5 mg. of cortisone. After 40 days, the tumors weighed only about 0.7 gm. in the treated animals, in contrast to 15 gm. in control animals. Despite this difference in the sizes of the transplanted tumors, there were no metastases in the control animals, but 10 of the 25 cortisone-treated mice had metastases in various organs. Using one of the metastases as a source of tumor

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cells for transplantation, Agosin *et al.* repeated the experiment, with similar results.

A report of the Subcommittee on Steroids and Cancer of the American Medical Association, in 1951 (*4*), included this statement concerning the use of cortisone in patients with advanced breast cancer: "Despite the subjective response and improved appearance of the patient, there was evidence of more rapid spread of the neoplasm and autopsy later revealed that even the spleen was riddled with metastases."

Until recently, we had not seen any clinical reports concerned with the chemotherapeutic use of cortisone in which the possibility of an adverse effect of cortisone on the growth of breast cancer or other malignancies has been mentioned. One paper in which this phenomenon is specifically discussed will be summarized later.

Molomut *et al.* (*5*) reported that a sarcoma which had originated in strain A mice grew in C57BL/6Jax mice pretreated with lyophilized tumor plus cortisone.

Gottschalk and Grollman (*6*) treated transplantable mammary carcinoma with cortisone or corticotropin (ACTH) *after* implantation, and reported that, with large doses of cortisone, tumor growth was inhibited and survival of the animals was prolonged.

Pomeroy (*7*) confirmed the previous observations that cortisone-treated mice developed significantly more metastases, and described the liver as the most frequent site of extrapulmonary metastases. When either Krebs-2 or Sarcoma 37 tumor cells were injected into the left ventricle, the preponderance of metastases in the cortisone-treated animals was enormous. Pomeroy theorized that perhaps cortisone inhibition of local host factors and depression of reticuloendothelial tissues resulted in a failure of tissue-specific antibody production.

Baserga and Shubik (*8*) began cortisone treatment of DBA mice both before and after implantation of a DBA tumor. Control mice developed no metastases; 8 of 22 mice given injections of cortisone 10 days *before* the tumor implant developed metastases. In those in which cortisone injections were delayed until 6 days *after* implantation, there were 5 metastases in 8 animals; in a third group, in which treatment was started 15 days after tumor implantation, there was only 1 metastasis in 18 mice. These investigators also demonstrated an enhancement of metastases from original tumors induced with methylcholanthrene.

In a second paper, Baserga and Shubik (*9*) described experiments in which tumor transplants in the tails of mice were subsequently excised, and half of the animals were treated with 0.5 mg. of cortisone on alternate days for a total of 4 or 5 injections. When the animals were examined 2 weeks later, there were 56 metastases in 38 control mice, and 193 metastases in 38 cortisone-treated mice. This experiment indicates that the enhancement of metastases by cortisone is not limited to a single tumor-host relationship, and emphasizes the earlier observations by these investigators that cortisone seems to affect the local milieu of the disseminated tumor cells.

Similarly, Duran-Reynals (*10*) found that cortisone induced metastases from a tumor that ordinarily did not metastasize. He suggested that the action of cortisone on tumor cells and the host was comparable to its effect on certain slow-growing bacterial cells.

Contrary evidence concerning the role of cortisone in the growth of tumors (*11–14*) and metastases (*15*) has been reported.

Wood *et al.* (*16*) studied the influence of pituitary growth hormone on the number of lung metastases developing after intravenous injection of Sarcoma T241 cells. Pretreatment of the recipient mice was followed by increased tumor growth in the lung and an accelerated growth rate. Growth hormone given after cell injection was ineffective. A parallel experiment, in which growth hormone was given for 12 days to mice with subcutaneous tumor implants, did not result in an increase in lung metastases.

Watson (*17*) reported that a series of cortisone injections, after inoculation of Ehrlich ascites cells into the peritoneal cavity, resulted in an inhibition of tumor growth.

Our paper reports attempts to assay the effect of various carcinostatic agents on cancer cells injected intravenously into mice—the effect of the agents before the cells could become "established." The experimental results were generally negative if a model system was such that the solid tumor from which the cells were derived was resistant to the chemical agent, *e.g.*, Ehrlich cells with tris(1-aziridinyl)phosphine sulfide (TSPA).

Kondo and Tsukui (*18*) had previously demonstrated the effect of toxic carcinostatic agents on host factors. Therefore, we wished to assay chemotherapeutic effectiveness when host factors were reduced by operative stress, chemical toxicity, and cortisone. Further, to detect the smallest possible number of tumor cells in the blood or in organs, either recipient mice had to be pretreated with cortisone, or inoculations had to be intracranial (*19*). The latter technique is excellent, but has certain practical disadvantages, hence the intensive studies of the effect of cortisone on the transplantation of cells.

MATERIALS AND METHODS

The tumors used in these studies were Krebs-2, Ehrlich (hyperdiploid), and Sarcoma 180 (S-180) ascites tumors obtained from T. S. Hauschka and carried in cousin-mated Swiss mice. Ascites cells were suspended in mammalian Ringer's medium and were counted in a Spencer-Neubauer hemocytometer. Only ascites cells from mice inoculated no longer than 7 days previously were used, to keep cell clumps and dead cells to a minimum. The cell suspensions were kept in ice water before and during the experiment; none were used after 2 hours, because of decreasing viability.

Only macroscopic metastases on the surface of the lungs and the liver were recorded. The maximum recorded was 10 metastases per lobe.

In instances where there was doubt as to the nature of a nodule, microscopic sections were prepared and examined.

The tail veins were used for all intravenous cell injections. Intracranial cell inoculations were performed by inserting a short #25-gauge needle through the sagittal sinus and aiming the needle point slightly to one side. The volume of such injections was never more than 0.05 ml.

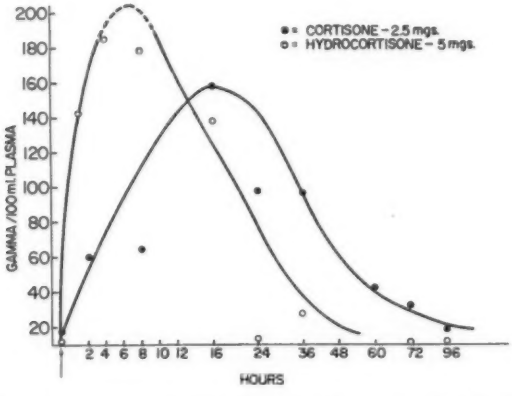
Cortisone acetate was injected subcutaneously, and hydrocortisone sodium succinate intravenously. The approximate blood levels attained and their durations are depicted in text-figure 1.

C3H mice with spontaneous mammary tumors were obtained from L. C. Strong. Induced tumors were provoked in Swiss mice by subcutaneous injection of 1.0 mg. of 3-methylcholanthrene in 0.1 ml. of sesame oil when the animals were 6 to 8 weeks old.

RESULTS

Time Relationship of Cortisone Treatment and Cell Inoculation

Text-figure 1 is derived from a single experiment and therefore represents an approximation of the blood levels attained after subcutaneous injection of cortisone acetate and hydrocortisone. Additional experiments gave similar results.



TEXT-FIGURE 1.—Blood levels of cortisone and hydrocortisone after subcutaneous injection.

A series of experiments to determine the optimal dosage, dosage schedule, and site of cell inoculum for assay of the smallest number of cells is outlined in tables 1 to 3.

The day of cell inoculation is designated as "zero" time. Cortisone injections given after cell inoculation are indicated by positive numbers of days, and cortisone injections given before cell inoculation are indicated by negative numbers of days.

Table 1 summarizes an experiment to determine the simplest, most effective dose of cortisone, and the optimal interval of administration before inoculation of small numbers of tumor cells into subcutaneous tissues.

TABLE 1.—Comparison of cortisone dosage and dosage schedules

Cortisone dosage			Mice with tumors/total No. of mice		
Amount (*mg.*)	Number of injections	Days	10*	100*	1000*
5	1	−1	12/23 (177)†	16/16 (322)	8/8 (843)
2.5	1	−1	16/23 (177)	16/16 (283)	8/8 (894)
2.5	2	−2, −1	10/22 (203)	15/15 (492)	8/8 (1103)
2.5	3	−3, −2, −1	7/22 (205)	11/16 (571)	8/8 (1227)
1.0	3	−3, −2, −1	7/23 (251)	11/16 (335)	8/8 (1031)

*Number of Ehrlich ascites cells inoculated subcutaneously.
†Numbers in parentheses indicate average weight (mg.) of tumors on day 30.

The mice were killed 30 days after inoculation with tumor cells; the numbers of mice with tumor growth and the weights of the tumors were recorded.

Table 2 indicates the importance of the relationship of the time of cortisone treatment to the time of intravenous cell inoculation in determining the subsequent establishment of metastases.

TABLE 2.—Ehrlich cells injected intravenously in mice; single injection of 5 mg. of cortisone subcutaneously

	Mice with tumors/total No. of mice	
Day of cortisone injection	Inoculated with 100,000 cells	Inoculated with 50,000 cells
−3	—	3/8
−2	4/6	3/8
−1	—	4/8
0*	2/7	0/8
+1	1/6	0/8
+3	0/8	0/8
+5	0/7	0/8

*Day of cell injection.

When tables 1 and 2 are compared, the importance of the inoculum site is evident; the subcutaneous tissues were the optimal site for tumor growth from the least number of cells. As will be mentioned later, the brain and the anterior chamber of the eye are excellent sites for assaying small numbers of cells, but are impractical because of the number of incidental deaths from injection trauma and infection.

Table 3 illustrates the same important time relationship with a greater number of cells.

TABLE 3.—Mice receiving 1×10^6 Ehrlich cells intravenously and 2.5 mg. of cortisone

Day of cortisone injection	Mice with tumors/ total No. of mice
−1	9/16
0	1/15
+1	0/16
+2	1/16
+4	0/16
+7	2/16
+10	1/16

Effect of Cortisone Pretreatment on Survival of Tumor Emboli

The effectiveness of cortisone in promoting the establishment of metastases in comparison with similar cell inoculums in normal animals is indicated in table 4. The term "metastasis" is used to describe surviving tumor emboli resulting from the injection of tumor cells intravenously. This conforms with previously published papers; the probable differences of these separate phenomena are acknowledged.

TABLE 4.—Comparison of "metastases" of Ehrlich cells in normal mice and in mice *pretreated* with various doses of cortisone*

	Site of cell inoculation†					
	Intravenous		Intraperitoneal		Subcutaneous	
Number of cells	Control	Cortisone	Control	Cortisone	Control	Cortisone
2,000,000	10/64					
1,000,000	7/78	7/7				
750,000		6/6				
500,000	2/24	11/13			8/8	
100,000	1/16	7/7	28/37	27/34	96/102	19/20
50,000		14/16				
25,000		7/8			15/16	
10,000		9/12	12/40	30/40	28/82	35/35
5,000	3/12	10/14			11/16	12/12
1,000	0/12	2/10	5/40	9/16	6/38	50/53
500						10/12
100			0/30	13/34	2/38	128/218
10			0/16	1/30	1/33	37/105
5					0/16	36/48
3					0/16	21/48
1					0/16	21/48

*Combined experiments.
†Mice with tumor growth / Number of mice inoculated

The growth of several other mouse ascites tumors in cortisone-pretreated animals was compared. As noted in the "Discussion," not all tumors are as resistant as these to the action of cortisone. Only by using relatively cortisone-resistant tumors can the effect of cortisone on host resistance be separated from its deleterious effects on the cells.

Table 5 compares the effectiveness of cortisone on the growth of "metastases" from Krebs-2, S-180, and Ehrlich ascites cells injected into a tail vein.

TABLE 5.—Comparison of effect of cortisone pretreatment on metastatic growth in mice inoculated intravenously with Krebs-2, S-180, or Ehrlich ascites cells

	Krebs-2*		S-180*		Ehrlich*	
Number of cells	Control	Cortisone	Control	Cortisone	Control	Cortisone
2,000,000			6/16		10/64	
1,000,000	4/32	22/24	2/32	8/8	7/78	7/7
750,000				8/8		6/6
500,000	7/45	7/8	3/32	6/8	2/24	11/13
250,000			2/32	7/8		
100,000	0/8	6/8	0/16	13/15	0/16	7/7
50,000	0/8	6/8	0/14	9/16	0	14/16
10,000	0	6/16	0			9/12
5,000		5/16				10/14
1,000		1/16	0/8	3/8		
500		0/16				

*Mice with tumor growth / Number of mice inoculated

Assays of Organs for Viable Tumor Cells

Cortisone-treated animals have also been used for assaying tumor cells in the blood and in various organs, subsequent to intravenous inoculation. The techniques have been previously described by Goldie *et al.* (*20*). A preliminary experiment was performed to evaluate the possible effect on transplantation rate when the blood is used as a suspension medium. Several such studies confirmed our impression that serum or blood is a better medium for preserving the viability of the tumor cells than any of the balanced salt solutions. Tissue culture medium supplemented with 10 percent serum is also excellent.

The organ distribution of metastases in cortisone-treated mice was given individual attention. In the treated mice, almost all metastatic lesions were in the liver, whereas untreated mice developed metastases in the lungs and only rarely in the liver.

In both groups of animals, histological studies revealed large numbers of tumor cells distributed in the lung veins, capillaries, and interalveolar tissue, and extruded into the alveoli themselves. Few tumor cells were detected in the liver sinusoids in either the control or experimental animals. Yet, as table 6 shows, as few as 50,000 Ehrlich cells injected intravenously resulted in massive, lethal, multiple liver metastases in 10 to 30 days. In contrast, consistent metastases in untreated mice necessitated the intravenous injection of 750,000 to 2 million Ehrlich cells. The metastases that develop in such untreated animals occur in the lungs and often do not attain sufficient size to cause death until 40 to 80 days, depending on the size of the cell inoculums. The paucity of metastases in other organs and the absolute absence of brain metastases suggested a need for early assays of various organs for the presence of tumor cells.

These observations of an apparent change in the organ distribution of metastases in cortisone-treated animals were rechecked by comparing the effects of a single dose of cortisone at various times before and after cell injection.

TABLE 6.—Distribution and number of metastases developing after intravenous inoculation with 50,000 Ehrlich cells

Cortisone (2.5 mg.) injected subcutaneously (*days*)	Conditioned mice*		Untreated mice
	Lung metastases	Liver metastases	
−4	1/8	6/8	No metastases in 64 animals
−3	0/8	3/8	
−2	3/15	10/16	
−1	0/16	11/16	
0†	0/16	2/16	
1	0/16	0/16	
2	0/8	0/8	
3	0/8	0/8	
4	0/8	0/8	

*Mice with tumor growth / Number of mice inoculated.
†Day of cell injection.

No lung metastases were found except in animals with practically complete replacement of the liver with tumor. These observations concerning the relative frequency of lung and liver metastases in conditioned mice were confirmed by an experiment in which the size of the cell inoculums of Krebs-2 or Ehrlich cells was varied (table 7).

TABLE 7.—Frequency of lung and liver metastases in conditioned mice receiving intravenous injections of Ehrlich or Krebs-2 cells

Cell inoculums	Ehrlich cell metastases*		Krebs-2 cell metastases*	
	Lung	Liver	Lung	Liver
1,000,000	1/7	6/6	2/5	2/5
750,000	1/6	5/6+	3/7	2/7
500,000	0/5	4/5	5/7	5/7
100,000	0/7	6/7	1/8	1/8
50,000	0/8	7/8	1/8	5/8
25,000	1/8	7/8	2/6	4/6
10,000	0/12	9/12	3/6	6/16
5,000	0/14	10/14	1/16	5/16

*Mice with tumor growth / Number of mice injected.

An additional experiment involving a large number of tumor cells and fractionated doses of cortisone resulted in a similar increased frequency of liver metastases, lung metastases occurring only in animals with massive replacement of the liver with tumor (table 8).

Review of several hundred untreated mice receiving intravenous injections of as many as 3×10^6 cells revealed the occurrence of liver metastases only rarely—usually in mice with widespread tumor growth.

Organ Distribution of Tumor Cells

The failure of the tumor cells to establish metastases throughout the animals receiving intravenous injections of tumor cells was striking. Presumably either the tumor cells were able to pass through the organs

TABLE 8.—Distribution of metastases in mice pretreated or posttreated with cortisone and inoculated intravenously with 1×10^6 Ehrlich cells

Treatment	Conditioned mice*		Untreated mice
	Lung metastases	Liver metastases	
Cortisone on days −4, −3, −2, −1			
2.5 mg. × 4	16/53	34/53	5/67
1.0 mg. × 4	16/58	17/42	(lung metastases)
0.5 mg. × 4	8/38	3/38	
Cortisone on days +1, +2, +3, +4			
2.5 mg. × 4	14/59	7/59	
1.0 mg. × 4	10/69	2/69	0/67
0.5 mg. × 4	3/39	0/39	

*Mice with tumor growth / Number of mice inoculated

that were not the sites of metastases, or else the cells were destroyed locally in the capillaries or the tissues. To study this question, tumor cells were injected intravenously. At intervals, the blood and the brei of separate organs were assayed for viable tumor cells.

Cortisone-conditioned animals and subcutaneous injection sites were used for these assays so that a minimal number of tumor cells could be injected. The blood was removed by aspiration of the right ventricle, both for assay purposes and to reduce the amount of blood in the various organs. The vascular system was not flushed with saline. A subcutaneous injection of 1 ml. of blood, or of organ brei consisting of either one kidney, both lungs, a liver lobe, one brain hemisphere, or an equal volume of skeletal muscle of the hind leg, was given at the neck. Previously, cortisone had been injected subcutaneously at the groin.

Two preliminary experiments are summarized in tables 9 and 10.

The recovery of viable tumor cells from the blood as long as 72 hours after injection is remarkable. Equally notable is the finding of tumor cells in the liver, even though metastases rarely develop in mice not treated with cortisone. Although one could tentatively conclude that the tumor cells are distributed to most organs, these data do not afford proof that the tumor cells remain in the organs permanently, but are destroyed *in situ* and thus are not revealed by these assay techniques. In future experiments, isotope-tagged cells should be used in an effort to resolve this dilemma.

Two series of *donor* mice were treated with cortisone before and after intravenous cell inoculation. The blood and various organ brei from these mice were assayed for the presence of tumor cells by injection into conditioned recipient mice. No apparent difference in cell survival or distribution was evident between these mice and untreated donor mice except for a persistence of tumor cells in the circulating blood.

ACTH was tested for its effect on the survival of Ehrlich cells injected subcutaneously and intravenously. The injection of one unit of ACTH 24 hours before cell inoculation was not as effective as an injection of

TABLE 9.—Assay of blood and organ brei after to intravenous injection of 2×10^6 Ehrlich cells*

Organ	Hours after injection:				
	1	2	4	6–8	24
Blood	3/3	3/3	8/10	8/9	1/10
Brain	0/3	0/3	0/10	0/9	2/10
Lungs	3/3	2/3	5/10	5/9	5/10
Liver	—	—	2/10	0/6	6/10
Muscle	0/3	0/3	3/10	0/9	1/10

*Subcutaneous assay in cortisone-conditioned mice.

TABLE 10.—Assay of blood and organ brei after intravenous injection of 1×10^6 Ehrlich cells*

Organ	Hours after injection:					
	1	2	4	6–8	24	72
Blood	3/3	1/3	5/11	9/10	2/10	2/4
Brain	2/3	0/3	5/11	0/10	1/10	0/4
Lungs	2/3	0/3	10/11	4/10	1/10	2/4
Liver	+–	—	4/11	2/10	5/10	4/4
Muscle	1/3	0/3	4/11	0/10	1/10	0/4

*Subcutaneous assay in cortisone-conditioned mice.

2.5 mg. of cortisone, but did increase the frequency and growth rate of metastases.

DISCUSSION

These studies are not necessarily pertinent to other transplantable cell lines or spontaneous tumors, and hence cannot be considered directly relevant to clinical phenomena. The importance of variations in techniques on experimental results was clearly evident in these studies concerned with isolating, counting, and injecting small numbers of cancer cells.

These variables include the individual characteristics of the ascites cells, such as their resistance to cortisone, their ability to survive as single cells, the "age" of the ascites used as the source of cells, the suspension medium, the manner of handling the cells at the proper temperature with minimal time *in vitro*, the age and sex of the assay animals, and, of course, the genetic background of the animals. All of the reservations concerning the use of transplanted tumor cells are pertinent.

Nevertheless, these experiments are inherently valuable. Certain phenomena have been observed; few are new observations, but these experiments have revealed new facets:

1. Single Ehrlich ascites cells will survive in cortisone-conditioned mice. Single S-180 and Krebs-2 cells also will probably survive in conditioned mice, but our data are not complete.

2. Single cells will exist and grow when injected directly into the brain, but exposure of the brain to thousands of tumor cells circulating in its blood vessels does not result in a single metastasis.

3. Cell survival varies at different sites. In both conditioned and unconditioned animals, subcutaneous tissue is the optimal site for the growth of a minimal number of cells, the peritoneal cavity is another good site, and the blood stream is a third.

4. Viable Ehrlich cells can be recovered from the blood stream as long as 72 hours after an intravenous injection of 1×10^6 cells.

5. The preferential site of metastases is the lungs in normal animals, and the liver in cortisone-treated animals.

All three types of ascites tumor cells, Ehrlich (hyperdiploid), Krebs-2, and S-180, are relatively insensitive to cortisone. This insensitivity made possible the independent study of the effect of cortisone on host resistance. It is unlikely that these results could have been duplicated with all types of tumors. For example, the cell growth of lymphosarcomas and rhabdomyosarcomas is so greatly inhibited by cortisone that a concurrent depression of host resistance could not be demonstrated. Similarly, we have induced tumors with 3-methylcholanthrene injections in the hope of evaluating a possible alteration of growth rate and ability to establish metastases if the host was treated with cortisone. Unfortunately, the sarcomas that have developed are markedly inhibited by cortisone, and thus a valid experiment is impossible.

The Walker carcinosarcoma is another example of a cortisone-sensitive tumor. Pretreatment of rats inhibits the growth of this tumor about 50 percent (*21*). Contrariwise, Fisher and Fisher (*22*, *23*) were able to influence host resistence to this tumor by means of surgery.

The host resistance of mice can be similarly reduced by sham abdominal operations, but this method is neither as convenient nor as effective as cortisone injections. For example, if experimental celiotomy is performed 4 hours before 500,000 Krebs-2 cells are injected into the tail vein, 83 percent of the animals developed lung metastases, in contrast to a frequency of 34 percent in the controls.

The suitability of the brain for the growth of small numbers of cells and for certain heterotransplants is well known. Prince (*19*) has performed excellent experiments designed to show the effectiveness with which 1 to 10 Ehrlich, Krebs, and S-180 cells can be assayed by intracranial inoculation. This technique requires mice no older than 8 weeks, to avoid brain trauma from deformation of the ossifying skull. The volume of the inoculums should be less than 0.05 ml., a limitation which is an additional handicap. The method is valid but much less practical than the use of cortisone-treated mice.

Pregnant mice are slightly better hosts for the growth of small numbers of cells, but the magnitude of the effect of this condition on malignant cell growth is small. Relevant experiments will be reported in detail in a future paper.

Pretreatment with irradiation also favors the growth of transplanted cells. This subject has been adequately reviewed elsewhere. We our-

selves have found chemical inhibition of growth-restraining factors to be simpler.

Kondo and his associates (*18*, *24*) have demonstrated that a number of carcinostatic chemicals have adverse effects on host resistance. If these compounds are toxic and the tumor is insensitive to them, their use results in an increased rate of tumor growth and of metastatic spread. This effect is best demonstrated by pretreating the animals with the drug before cell inoculation.

Kondo and Tsukui (*18*) have shown that variations in tumor growth and metastasis production may result from the treatment of animals inoculated with tumor sublines, one of which is sensitive and another insensitive to a single drug. These investigators suggest that these toxic agents may not only affect host resistance but also increase the exfoliation of cells from the tumor by reducing cellular adherence.

Laszlo, Burk, and Wight (*25*) have recently reported that mice pretreated with X ray, heat stress, formalin, Clorpactin, and methotrexate had an enhanced growth of Krebs-2 ascites cells injected into the peritoneal cavity. The doses used of Clorox and hydrocortisone inhibited cell growth. Similar results were found when a lymphoid tumor originating in the test strain was used. They also demonstrated the adverse effects of supraoptimal doses of hypochlorite and the critical importance of time-dose relationships.

The role of the reticuloendothelial system in the experiments described in this paper is unknown. Preliminary experiments in which India ink was used to block the reticuloendothelial cells did not affect either the frequency or distribution of metastases in normal and conditioned animals. O'Brien *et al.* (*26*) have reported that blockage of the reticuloendothelial system with ink particles diminishes capillary-resistance response to cortisone. The pertinence of this observation to the present work cannot be evaluated at present.

The observation that pretreatment with cortisone causes a shift in the organ distribution of metastases is extremely important. This is clear evidence that a difference in "soil" exists (*27*, *28*) and can be altered. The local factors in the liver, muscle, and brain that normally inhibit the development of metastases require further study. Further, these phenomena are different, as is demonstrated by the marked alteration in the susceptibility of the liver as a site for metastatic growth by cortisone and the apparently unchanged adverse environment of skeletal muscle and brain. One might predict that in addition to host resistance related to orthodox immunological factors (*e.g.*, antibody production, and active destruction of tumor cells by phagocytic elements), there may be antagonistic reactions of even greater importance between host cells and tumor cells.

Although the experiments reported in this paper are not applicable to human malignancy, it is appropriate to mention several pertinent articles. Pelner (*27*, *28*) has reported an apparent association between cortisone treatment and enhancement of the growth of several kinds of malignancy.

Similarly, Iversen and Hjort (*29*) noted an increase in the frequency of breast-cancer metastases to the spleen in patients treated with cortisone or prednisolone, or both. There was no obvious difference in the metastases in other organs. At the least, this observation is further evidence that perhaps the "soil" for metastases can be altered.

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Synthetic Auxins in the Chemotherapy of Transplanted and Spontaneous Mouse Adenocarcinoma [1]

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SUMMARY

Methyl esters of certain synthetic auxins such as 2,5-dichlorophenoxyacetic acid and 2,3,4,5-tetrachlorophenoxyacetic acid, as well as the related compound, 2,4-dichlorophenol, were found to inhibit the rate of growth of adenocarcinoma H2712J and of spontaneous breast adenocarcinoma in mice. These agents do not affect the bone marrow in doses which are carcinostatic. Synthetic auxins and polychlorophenols, therefore, are a new family of compounds suitable for study in cancer chemotherapy.—J. Nat. Cancer Inst. 25: 1111–1120, 1960.

INVESTIGATION OF synthetic auxins (plant growth regulators) for their carcinostatic activity has been undertaken for the following reasons: In adequate doses, some of the auxins exert selective growth inhibition in plants that is most intense in the youngest and least-differentiated tissues. They inhibit tumoral plant tissues of crown galls *in vitro* at lower concentrations than the corresponding normal tissues (*1–5*). Outstanding metabolic properties of auxins at plant-growth-inhibiting concentrations are acceleration of the catabolism of carbohydrates (*6, 7*), lowering of cellular potassium content (*8*), and, apparently, increase of adenotriphosphatase (ATPase) activity (*9–11*). Plant-growth-inhibiting concentrations of such auxins also depress the levels of nucleic acids within the cells (*12*); they antagonize adenine (*13*), promoters of cell division such as the "kinins" of coconut milk (*14*), and 6-furfurylaminopurine or "kinetin" (*15*).

Auxins have repeatedly been active in animals and in animal tissue cultures (*16–21*). However, no tumor-inhibiting activity, with auxins which were used as free acids and salts, was observed in domestic animals and human beings. 2,4-Dichlorophenoxyacetic acid acts mostly as a

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nondissociated molecule and progressively loses activity as it is increasingly ionized with rising *p*H, as demonstrated by Audus (*22*). Since the *p*H of blood and tissues in mammals is 7.3 to 7.4, and thus far above the *p*H of the interior medium in plants (5.5–6.0), it was decided to experiment with nonionizable auxin derivates such as esters, which also possess plant-growth-regulating properties.

Because of some encouraging preliminary observations made with such esters in patients (*23*) and domestic animals (*24*), it was decided to evaluate more systematically the tumor-inhibiting properties of these and related compounds in transplanted and spontaneous mouse tumors. Polychlorophenols, which in plants are transformation products of chlorine-substituted phenoxyacetic acids (*25–28*), and are known to increase their herbicidal activity, were also studied.

Pilot experiments indicated a lack of carcinostatic activity of indole-3-acetic acid methyl ester, 2,4-dichlorophenoxyacetic acid methyl ester, 2,5-dichlorophenoxyacetic acid methyl ester, 2,4,5-trichlorophenoxyacetic acid methyl ester, 2,4,6-trichlorophenol for Ehrlich's ascites tumor, and of 2,5-dichlorophenoxyacetic acid methyl ester for Sarcoma 1 in A/Jax mice. In contrast, regression of tumor growth was observed in spontaneous breast cancers of C3H mice after treatment with 2,4-dichlorophenol, 2,5-dichlorophenoxyacetic acid methyl ester, and 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester, and in transplanted breast adenocarcinoma H2712J in C3H and (DBA/2 $\times$ C3H)F_1 mice after treatment with these same two esters.

EXPERIMENTAL

Male and female mice,[4] 8 to 10 weeks old, were used in these studies. They were kept in air-conditioned animal rooms at temperatures ranging from 70 to 74° F., 5 to 10 to a cage, with wood shavings as bedding and Purina chow and tap water given *ad libitum*. At least 20 animals were used for each experiment, except for studies with spontaneous tumors in which 10 to 12 animals were used per group. The animals were weighed at the beginning of each experiment and again at the time of autopsy. Tumor transplants [4] were made by the Snell (*29*) cytosieve method, and tumor suspensions of 8 to 13 percent cell volume in Ringer's solution were used. Subcutaneous tumor injections were made with 0.1 ml. of these suspensions and were given alternatively to control animals and animals to be treated so that each group received tumor-cell suspensions of comparable age. Tumor weights were obtained at the end of each experiment, and representative samples of tumors were fixed in 8 percent formaldehyde or Bouin's solution and stained with hematoxylin-eosin for histologic examination.

The mammary-gland adenocarcinomas [4] of C3H females of varying ages were measured by means of a new "plastogram" method described

[4] All tumors and mice used in these studies were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

in the next paragraph. Animals with tumors of comparable size were selected for control and treated groups, and plastograms of the tumors were made at weekly intervals. At the end of the experiments on spontaneous tumors, the animals were killed and tumors prepared for histologic study.

Plastograms were obtained by the following procedure: The animals were anesthetized with Nembutal and placed so that a plastic sheet could easily be placed over the tumor. The outlines of the animal and tumor were drawn on the plastic with a soft-marking pencil. The tumor outline was cut out and weighed. The weight obtained was divided by the weight of 1 cm.^{2} of identical plastic, and the resulting figure approximated tumor cross-sections area in cm.^{2} The excised tumor area of the plastogram may be reinserted into the plastic sheet which is kept for future comparison with subsequent plastograms. This method is reproducible by independent observers and provides not only accurate figures but also a visual record of tumor growth in intact animals.

As indicated in "Results," daily injections of the test substances were given subcutaneously at sites distant from the tumors for from 6 to 37 days, and the doses varied from 0.5 mg. per day to 2 mg. per day per animal. Control animals received the appropriate solvents alone, and "positive" controls were made with 6-mercaptopurine and 8-azaguanine [5] as known tumor inhibitors. Toxicity studies were carried out with 2,5-dichlorophenoxyacetic acid methyl ester by the administration of this substance in pellets subcutaneously into Swiss mice. The residual substance was weighed 3 to 7 days after implantation. The survivors were killed and histologic studies were made on all organs, including bone marrow. Animals dying during toxicity experiments were autopsied and studied histologically.

The toxicity of 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester was determined in 30 Swiss mice at dose levels of 0.5, 1, 2, 4, and 8 mg. subcutaneously for 7 days in groups of 6 animals each.

Substances tested and solvents used are listed in tables 1 and 2. The substances listed in table 1 yielded negative or borderline significant results in the systems indicated.

Statistical evaluations were made of the tumor weights at the end of the experiment. The data of the control (solvent or vehicle) animals were compared with those obtained in animals having received the test substances and these data were given the t test for significance. The significance of a difference has been derived from t distribution tables at a confidence level of 1 percent.

RESULTS

The results are tabulated in tables 1 and 2 and text-figure 1.

[5] 6-Mercaptopurine was obtained through the courtesy of Dr. G. H. Hitchings, of the Burroughs Wellcome Laboratories, Tuckahoe, New York; 8-azaguanine was kindly supplied through Dr. C. C. Stock, of the Chemotherapy Division of the Sloan-Kettering Institute, New York.

TABLE 1.—Substances yielding significant inhibition of transplanted Adenocarcinoma H2712J

Substance	Dose (*per day*)	Vehicle or solvent	Route	Host (*number of animals*)	Ratio of tumor weights (*treated/controls*)	Average inhibition (*percent*)	*P*	*t*	Body-weight changes (*treated/controls*)	Deaths	
										Treated	Controls
6-Mercaptopurine	0.5 mg. × 7	0.5% Carboxymethylcellulose* in phys. saline	Intraperitoneal	(DBA/2 × C3H) F_1 female mice (24)	0.545	45.5	<0.01	7.8	+1.33/−0.2	4/24	0/24
8-Azaguanine	1.5 mg. × 7	5% Gum acacia in phys. saline	Intraperitoneal	(DBA/2 × C3H) F_1 male and female mice (24)	0.61	39	<0.01	5.5	−1.5 /+0.2	1/24	0/24
2,5-Dichlorophenoxyacetic acid methyl ester	1.0 mg. × 9	Cod-liver oil with 1% Span 80	Subcutaneous	C3H male mice (24)	0.636	36.4	0.01–0.025	2.2	−0.6/−1.52	3/24	3/24
2,5-Dichlorophenoxyacetic acid methyl ester	8.6 mg. × 7	Cod-liver oil with 1% Span 80	Subcutaneous	(DBA/2 × C3H) F_1 female and male mice (24)	0.77	23	<0.01	2.9	−1.9/−0.6	2/24	0/24
2,3,4,5-Tetrachlorophenoxyacetic acid methyl ester plus 2,4-dichlorophenol	0.5 mg. × 7 25γ	Glycerol tripelargonate	Subcutaneous	(DBA/2 × C3H) F_1 female mice (24)	0.74	26	<0.01	3.4	+0.5/−0.2	0/24	0/30
2,3,4,5-Tetrachlorophenoxyacetic acid methyl ester	2.0 mg. × 8	Glycerol tripelargonate	Subcutaneous	(DBA/2 × C3H) F_1 female mice (22)	0.50	50	<0.01	5.4	−3.2/+3.0	0/22	0/22
2,3,4,5-Tetrachlorophenoxyacetic acid methyl ester	1.0 mg. × 7	Glycerol tripelargonate	Subcutaneous	(DBA/2 × C3H) F_1 female mice (22)	0.48	52	<0.01	8.2	−1.0/+0.9	0/22	0/22

*Hercules, C.M.C. Type 70 High, Hercules Powder Co., Wilmington, Del.

TABLE 2.—Experiments showing weak (insignificant) or no tumor inhibition with compounds listed and in specific tumor-host systems indicated

Substance *(dose, mg./day × days)*	Solvent or vehicle	Host	Tumor
Indoleacetic acid methyl ester (2 mg. × 5)	Cod-liver oil with Span 80 *1%*	Swiss female mice	Ehrlich's ascites
α-Naphthaleneacetic acid methyl ester (2 mg. × 6)	Cod-liver oil with Span 80 *1%*	Swiss male mice	Ehrlich's ascites
2,3,6-Trichlorobenzoic acid (1 mg. × 7)	Glycerol tripelargonate*	(DBA/2 × C3H)F_1 male mice	Adenocarcinoma H2712J
2,4-Dichlorophenoxyacetic acid methyl ester (1 mg. × 10)	Cod-liver oil with Span 80 *1%*	C3H male mice	H2712J
2,5-Dichlorophenoxyacetic acid methyl ester (1 and 2 mg. × 7)	Cod-liver oil with Span 80 *1%*	Swiss male mice	Ehrlich's ascites
2,5-Dichlorophenoxyacetic acid methyl ester (1 mg. × 11)	Cod-liver oil with Span 80 *1%*	A/He female mice	Sarcoma I
2-Methyl-5-chlorophenoxyacetic acid methyl ester (1 mg. × 9)	Cod-liver oil with Span 80 *1%*	C3H male mice	H2712J
2,4,5-Trichlorophenoxyacetic acid methyl ester (2 mg. × 5)	Cod-liver oil with Span 80 *1%*	Swiss female mice	Ehrlich's ascites
2,3,4,6-Tetrachlorophenoxyacetic acid methyl ester (1 mg. × 7)	Glycerol tripelargonate	(DBA/2 × C3H)F_1 female mice	H2712J
2,5-Dichloroanisole (2 mg. × 8)	Glycerol tripelargonate	(DBA/2 × C3H)F_1 male mice	H2712J
2,5-Dichlorophenol† (2 mg. × 7)	Glycerol tripelargonate	Swiss male mice	Ehrlich's ascites

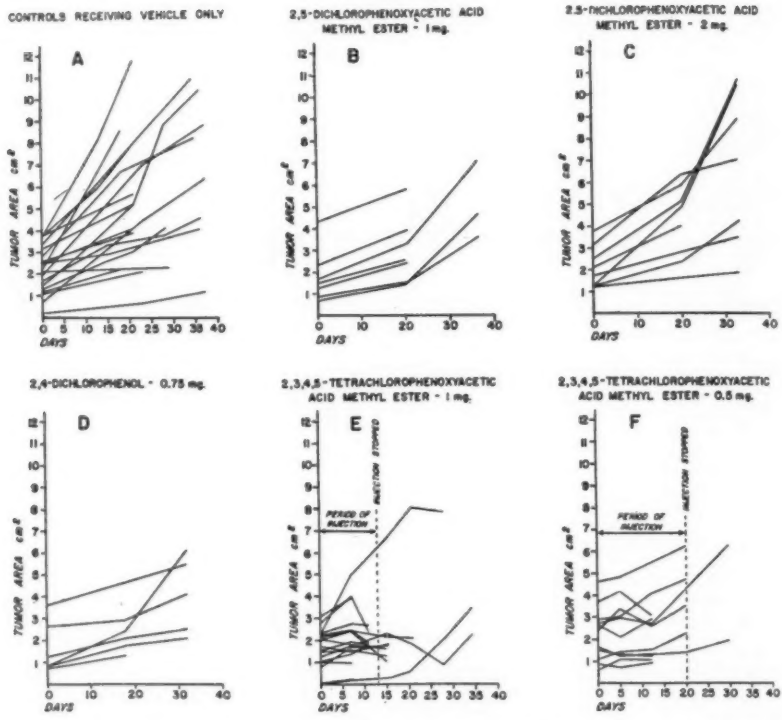
*The glycerol tripelargonate was supplied by Emery Industries, Cincinnati, Ohio.

†Nearly significant reduction of tumor weight and cell volume, significant reduction of cell concentration (cytocrit).

2,5-Dichlorophenoxyacetic acid methyl ester in subcutaneous pellets caused marked body-weight loss in Swiss mice within 2 days and amounts of the substance absorbed varied from 12 to 25 mg. per day. This was approximately the LD50, since 3 of 6 animals died after absorbing 15 to 19 mg. per day, or approximately 600 to 800 mg. per kg. of body weight per day. The doses causing a significant tumor inhibition were 1 to 2 mg. per day, or approximately 40 to 80 mg. per kg. per day. The LD50 of 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester in glycerol tripelargonate was approximately 4 to 8 mg. per day for 7 days or 160 to 320 mg. per kg. per day. Weight loss occurred with doses larger than 4 mg. per day but not with smaller amounts.

Weight loss, which was one of the early signs of toxicity, was most marked in young animals and least marked in fully grown ones. In this respect, the mammalian organism responded like the reaction of plants to such growth regulators: the younger the tissue, the more marked its response.

Growth curves of spontaneous breast adenocarcinoma in C3H mice are shown in text-figure 1. It is noteworthy that the depression of the growth



TEXT-FIGURE 1.—All experiments shown in this text-figure were done with C3H mice bearing spontaneous breast adenocarcinomas.

A. Control animals treated only with daily injections of tripelargonate. Mice died from tumor growth or were killed for histology.

B. Tumor growth curves of animals receiving 1 mg. of 2,5-dichlorophenoxyacetic acid methyl ester in cod-liver oil with 1 percent Span 80 daily for 37 days. Statistical significance of tumor growth inhibition: $P = 0.01$, $t = 3.2$.

C. Tumor growth curves of animals receiving 2 mg. of 2,5-dichlorophenoxyacetic acid methyl ester in cod-liver oil with 1 percent Span 80 daily for 34 days. Statistical significance of tumor growth inhibition: $P = 0.025$–0.05, $t = 2.1$.

D. Tumor growth curves of animals receiving 0.75 mg. of 2,4-dichlorophenol in cod-liver oil daily for 34 days. Statistical significance of tumor growth inhibition: $P = 0.01$, $t = 5.7$.

E. Tumor growth curves of animals receiving 1 mg. of 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester in glycerol tripelargonate daily for 14 days, when most of them were killed for histology. Statistical significance of tumor growth inhibition: $P = 0.01$, $t = 2.28$.

F. Tumor growth curves of animals receiving 0.5 mg. of 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester in glycerol tripelargonate for 14 days. Statistical significance of tumor growth inhibition: $P = 0.01$, $t = 3.04$.

curves seen during the first 20 days of treatment subsided thereafter, which indicated an escape phenomenon like that observed with other carcinostatic agents.

Histologic studies of animals killed by toxic doses of 2,5-dichlorophenoxyacetic acid methyl ester and 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester showed microscopic hemorrhages in the spleen, the lungs, and the bone marrow, and hemosiderin in the liver. There was no evidence of karyolytic effects upon hematopoietic cells, except in animals killed by the highest doses of 2,5-dichlorophenoxyacetic acid methyl ester. Mitotic activity in the intestinal mucosa and testes was not suppressed.

Histologic studies [6] of spontaneous adenocarcinoma of the mammary gland in C3H mice and of transplanted Adenocarcinoma H2712J reveal a certain degree of spontaneous, central necrosis occurring in untreated tumors. Treatment with auxins, especially 2,5-dichlorophenoxyacetic acid methyl ester, frequently, but not invariably, increased the degree of necrosis. Some regressing tumors showed marked connective-tissue proliferation from the periphery which resembled that seen in some estrogen-treated neoplasms. Surviving tumor cells usually showed some degree of mitotic activity.

DISCUSSION

The data presented here indicate that 2,5-dichlorophenoxyacetic acid methyl ester, 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester [7] alone and combined with 2,4-dichlorophenol, and 2,4-dichlorophenol alone exert a carcinostatic effect upon transplanted Adenocarcinoma H2712J and upon spontaneous breast adenocarcinoma in C3H mice.

In contrast, 2,5-dichlorophenoxyacetic acid methyl ester was without such effect in Ehrlich's ascites tumor and in Sarcoma 1. α-Naphthaleneacetic acid methyl ester also had no effect on Sarcoma 1, and 2,4-dichlorophenoxyacetic acid methyl ester, 2-methyl-5-chlorophenoxyacetic acid methyl ester, and 2,3,4,6-tetrachlorophenoxyacetic acid ethyl ester were without carcinostatic effect in Adenocarcinoma H2712J. These contrasting observations regarding 2,5-dichlorophenoxyacetic acid methyl ester indicate that Sarcoma 1 and Ehrlich's ascites tumor may be unsuitable screens for the study of carcinostatic effects of synthetic auxins and related compounds, with spontaneous breast adenocarcinomas having greater sensitivity for growth-inhibiting effects of these compounds.

The growth-depressing effect of these substances on *spontaneous* breast tumors is of special interest, since according to Furth (*30*) ". . . efforts to bring about regression of spontaneous tumors . . . have thus far invariably failed," and also in view of the observations of Sugiura *et al.* (*31*), showing that these spontaneous tumors were unaffected by 8-azaguanine.

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[6] The help of A. B. Russfield, M.D., Ph.D., in studying the histopathology of these experiments is gratefully acknowledged.

[7] 2,3,4,5-Tetrachlorophenoxyacetic acid methyl ester was not tested against S-1 or Ehrlich's carcinosarcoma, and the phenols alone were not tested against any of the transplanted tumors.

The results obtained with 6-mercaptopurine and 8-azaguanine in transplanted Adenocarcinoma H2712J confirm the inhibiting effects of these antimetabolites on solid tumors (*31*). In the systems used, their carcinostatic effect was similar to that of 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester. The latter brought about inhibitions and values of statistical significance slightly superior to those exhibited by 6-mercaptopurine which was itself more efficient than 8-azaguanine. At the doses used to induce carcinostatic effects, the toxicity of 2,4- and 2,5-dichlorophenoxyacetic acid methyl ester is low. For 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester, the zone of cytostatic activity is closer to the levels in which toxic symptoms are exhibited. However, in experiments on transplanted tumors, when it was given for 7 to 9 days, its toxicity does not appear to be much greater than that of 6-mercaptopurine and 8-azaguanine as shown by body-weight loss and deaths. In long-term experiments with spontaneous tumors, massive necrosis of the skin at and around the site of injection may have contributed to the death of some animals after 12 days of treatment.

Toxic symptoms brought about by the substances referred to are weight loss and, later, myotonia of the hind legs and a slight ataxia. Synthetic auxins at plant-growth-inhibiting doses are known to have a strong influence on cell permeability (*32*), and much of the weight loss may be ascribed to loss of water. Altogether, these symptoms correspond with those clinically observed in patients (*23*) receiving 2,4,5-trichlorophenoxyacetic acid isopropyl ester or 2,4-dichlorophenoxyacetic acid ethyl ester in doses of more than 1.25 gm. per day by intramuscular injection. In animal experiments, no evidence of bone marrow toxicity was obtained except with toxic doses exceeding the LD50. Even then, the toxic effects upon bone marrow were hemorrhages and not cell depletion.

The structural requirements for carcinostatic activity are, with the exception of the chlorinated phenols, similar to those established for plant-growth-regulating activity. This appears from the total lack of activity of the isomer 2,4,5,6-tetrachlorophenoxyacetic acid methyl ester and of 2,5-dichloroanisole. The latter induced the same average body-weight loss as 2,3,4,5-tetrachlorophenoxyacetic acid methyl ester, but without any tumor inhibition, which seems to indicate that there is more than an unspecific stress effect in the carcinostatic activity observed by us. Also, there has been, in at least one experimental group, a significant tumor inhibition accompanied by a weight gain as compared to a weight loss in the controls (table 1).

The absence of toxic effects upon the hematopoietic tissue, together with their carcinolytic effects in appropriate tumor-host systems and the preliminary clinical observations published earlier, suggests strongly that synthetic auxins are a new family of compounds that deserve intensive study to determine their possible place in cancer chemotherapy. They possess the marked differential potency inherent in the natural inhibition of plant growth emphasized by Garb (*33*).

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Formation of Ketopentoses, Beginning With Ribose-5-Phosphate, in the Serum of Patients With Cancer [1, 2]

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SUMMARY

We have studied the formation of ketopentoses, starting with ribose-5-phosphate, in the serum of 98 normal individuals, 15 patients with nonmalignant diseases, and 104 patients with malignant neoplasias in the different stages of evolution. The results indicated that the activity of the enzymes which intervene in the reaction tends to be greater in the serum of patients with malignant neoplasias, a tendency which becomes even greater in patients with advanced metastatic cancer. Serum of the group with acute leukemia showed evident difference from that of the control group, and gave higher maximum normal values. These results are discussed from the point of view of diagnosis and evolution of the malignant neoplasias.—J. Nat. Cancer Inst. 25: 1121–1126, 1960.

THE PRESENCE of some enzymes of the Warburg-Dickens pathway in the blood serum has been demonstrated by Bruns (*1*) and Guzmán-Barrón and coworkers (*2*). Bruns studied the activity of the ribose-5-phosphate (R-5-P) isomerase in the serum of healthy individuals, of patients suffering from malignant and nonmalignant diseases, and of several species of animals. Guzmán-Barrón *et al.* studied the disappearance of the R-5-P with the subsequent appearance of fructose and heptulose in the serum of healthy individuals, of women in the 9th month of pregnancy, and of patients with advanced cancer.

Both investigators found greater enzymatic activity in the serum of patients with cancer, but their findings were in small groups of patients and suggested the need for enlarging this study to include a greater number of patients in different stages of development. The purpose of this investigation is to establish the value which the determination of these enzymes might have as a diagnostic test or as an index of the development of the malignant neoplasias.

METHODS

Human subjects.—Three groups of individuals were studied: (*a*) controls consisting of 98 persons (47 women and 51 men) apparently in good

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[2] This work was supported by a grant from the Eli Lilly and Company Research Laboratories, Indianapolis, Ind.

health, whose ages varied between 5 and 62 years; (*b*) 15 patients (14 women and 1 man) with nonmalignant diseases, whose ages varied between 8 and 65 years; (*c*) 104 patients (76 women and 28 men) with malignant neoplasias in different stages of development, whose ages varied between 4 and 82 years and whose diagnoses were verified by histologic examination from biopsy specimens, surgical specimens, or at autopsy (table 1).

TABLE 1.—Classification of malignant and nonmalignant diseases of patients in studies

Malignant diseases	Number of cases	Malignant diseases	Number of cases
Chronic leukemia	2	Osteogenic sarcoma	1
Acute leukemia	10	Choriocarcinoma	1
Malignant lymphomas	6	Cancer of thyroid	7
Cancer of breast	27	Bronchiogenic carcinoma	1
Cancer of cervix	10	Cancer of pharyngeal vault	1
Cancer of endometrium	1	Cancer of larynx	1
Malignant stromatosis	1	Cancer of tongue	2
Cancer of vulva	1	Cancer of oral mucosa	1
Cancer of ovary	1	Cancer of lip	2
Liposarcoma	3	Cancer of paranasal sinuses	1
Bronchogenic cancer	3	Hepatoma	1
Cancer of bladder	1	Cancer of gallbladder	1
Malignant neurilemmoma	1	Cancer of stomach	7
Adrenal cancer	1	Cancer of rectum	5
Malignant melanoma	1	Cancer of cecum	1
Cancer of skin	1	Carcinomatosis	1
Total: 104			

Nonmalignant diseases	Number of cases
Benign diseases of breast	4
Uterine myoma	1
Pregnancy (4th and 7th months)	2
Chronic cholecystitis	1
Perforated peptic ulcer	1
Acute hepatitis	1
Papilloma	2
Lipoma	1
Desmoid	1
Osteoma	1
Total: 15	

Procedure.—The procedure used by Bruns (*1*) was followed: R-5-P was incubated in the presence of the serum at 37° C. for 10 minutes and the quantity of ketopentose formed was measured. Dische and Borenfreund's (*3*) technique for the determination of the ketopentoses was used. Bruns expressed his results as μmoles of ribulose-5-phosphate (Ru-5-P) [formed by the action of ribose-5-phosphate isomerase] based on the fact that the R_F of the reaction product coincides with that of Ru-5-P in the chromatographic system used (*4*). However, there is a possibility that some Ru-5-P may be transformed into xylulose-5-phosphate (Xu-5-P)—as a result of possible action of the epimerase of the

serum. Since it is not known whether Xu-5-P presents the same R_F in such a chromatographic system, our results will be expressed as μmoles of ketopentoses. The substrate used was ribose-5-phosphate (Schwarz and Co., Mount Vernon, N.Y.), and the barium salt was transformed into potassium salt before use.

Samples of venous blood were taken from an antecubital vein, with the subject under basal conditions, and the blood was transferred to a test tube kept in a container with ice. After the blood samples coagulated, they were centrifuged at 2000 rpm for 10 minutes. The serum was then decanted and kept in the refrigerator until the moment of incubation. In all cases this was begun within 2 hours after drawing the blood, though it was established that it can be kept in the refrigerator for 24 hours without loss of activity.

The incubation procedure was as follows: 0.25 cc. of serum without hemolysis, 0.50 cc. of glass-distilled water, and 0.50 cc. of 0.1 M tris (hydroxymethyl)aminomethane buffer at *p*H 7.4 were preincubated for 5 minutes in a bath at 37° C. Then 0.25 cc. of 0.01 M solution of R-5-P potassium salt was added and the solution was returned immediately to the bath. After exactly 10 minutes of incubation, the tubes were placed in a container with ice, and 1.50 cc. of 10 percent trichloroacetic acid was added. At the same time, a blank test was made to which the substrate was added after the trichloroacetic acid. The samples were centrifuged and the supernatant liquid was decanted. With an aliquot sample of this liquid, we carried out the reaction of the cysteine-carbazole of Dische (*3*). The intensity of the color was read at 540 μ in the Coleman spectrophotometer, and the ketopentose concentration was determined in accordance with its extinction coefficient (*1*).

The results are expressed as μmoles of ketopentoses formed in 10 minutes by the enzymatic activity of 0.25 cc. of serum (μmoles ketopentoses/0.25 cc. serum/10 minutes at 37° C.).

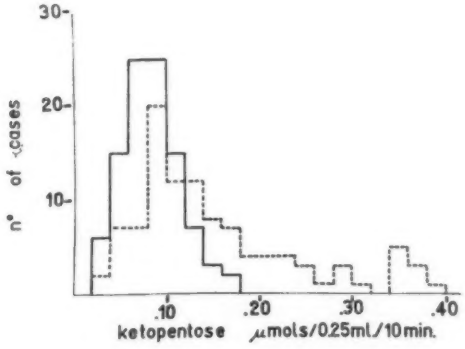
Our tests were made in serum, but it is possible to work with plasma treated with anticoagulants used in clinical practice at the customary concentrations (heparin, Wintrobe's mixture, potassium oxalate) without interfering with the results. The hemolysis that is seen with the naked eye interferes with the reaction because of the passage of enzymes of the red cells (*5*) in which they are found in greater concentration than in serum.

The alkaline phosphatase was not active on the ribose-5-phosphate, as already demonstrated by Bruns (*1*). This lack of activity was found even in serums with high phosphatase activity on β-glycerophosphate.

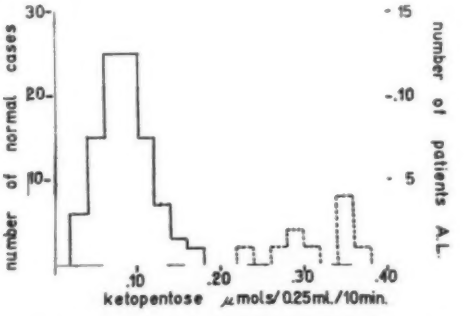
RESULTS

In the group of normal individuals there were no significant differences in relation to age or sex. The values varied between 0.03 and 0.180 with an average of 0.089 μmoles per 0.25 cc., for 10 minutes (0.031 S.D.).

The results found in patients with nonmalignant diseases were similar to those in normal individuals. The values varied between 0.07 and 0.190 with an average of 0.114 μmoles per 0.25 cc., for 10 minutes (0.031 S.D.). In the serum of patients with malignant neoplasias, values were obtained which varied between 0.020 and 0.390, with an average of 0.157 μmoles per 0.25 cc., for 10 minutes (0.091 S.D.). As can be seen in text-figure 1, the distribution curves of normal patients and of those with cancer overlap, and the latter show a definite tendency to give higher values. We found no relation between the type of tumor and the values obtained, except in the patients with acute leukemia (text-fig. 2) whose values were distributed above the normal ones. The neoplasias which presented metastases showed a tendency to give higher values, as shown in text-figures 3 and 4.



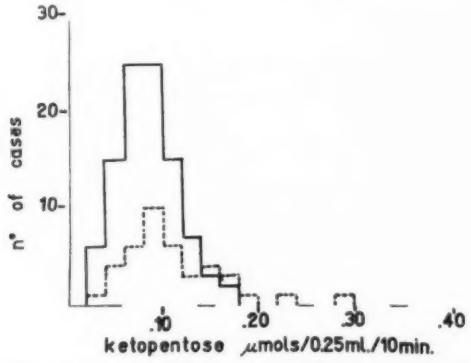
TEXT-FIGURE 1.—Distribution curve of ketopentose formation in normal serums (*solid line*) and in serums of patients with malignant neoplasias (*broken line*).



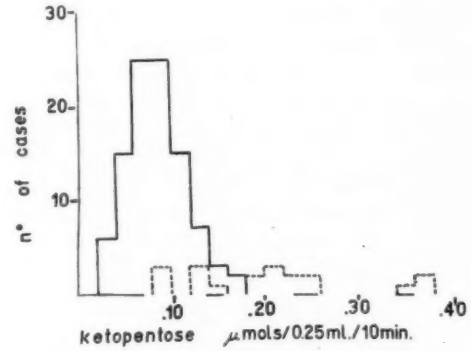
TEXT-FIGURE 2.—Distribution curve of ketopentose formation in normal serums (*solid line*) and in serums of patients with acute leukemia (*broken line*).

DISCUSSION

The group of patients with nonmalignant diseases, limited in number and variety of conditions, shows similar results to the group of normal



TEXT-FIGURE 3.—Distribution curve of ketopentose formation in normal serums (*solid line*) and in serums of patients with nonmetastasizing malignant neoplasias (*broken line*). Localized lymphomas are not included.

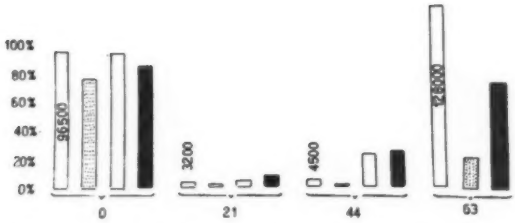


TEXT-FIGURE 4.—Distribution curve of ketopentose formation in normal serums (*solid line*) and in serums of patients with distant metastasis (*broken line*). Leukemias and lymphomas are not included.

subjects. Since Bruns (*1*) found high values in 2 of 3 cases of chronic nephritis and in some cases of uremia, liver cirrhosis, and chronic and acute hepatitis, it is evident that the increased activity in the formation of ketopentoses can be found in nonmalignant diseases.

Serum from the cancer patients tended toward greater activity in the formation of ketopentoses than that of the group which served as controls.

This tendency was more manifest in patients with metastasizing cancer, though the distribution curves of the values in normal and cancer patients always overlapped. It can be concluded that the procedure under study is not satisfactory as a diagnostic test for cancer. However, the patients with acute leukemia had serums whose activity was (in the 10 cases studied) above the maximum normal values. This finding, which apparently could be useful for the diagnosis of acute leukemia, loses diagnostic importance because this disease can be recognized by



TEXT-FIGURE 5.—Patients treated with Dexametasone (initial dose 12 mg. daily for 3 days, followed by decreasing dose by 0.5 mg. daily until dose of 4 mg. daily was reached). The changes in leukocytes in peripheral blood are shown (in the white column the total count per mm.^{3}), percentage of blastic cells in peripheral blood (*shaded column*), percentage of blastic cells in bone marrow (*white, clear column*), and enzymatic activity in serum (ketopentose formation, *black column*). Values shown were determined before treatment and at 21, 44, and 63 days after initial treatment. Ketopentose formation was 0.360, 0.033, 0.105, and 0.306 μmoles per 0.25 cc., for 10 minutes at 0, 21, 44, and 63 days, respectively.

methods which are safe, simple, and economical, as in the hematologic study of the circulating blood and of the bone marrow. It could, however, serve as a treatment evaluation index of acute leukemia, since we observed that in patients who improved with treatment the activity decreased to normal limits, then increased again when there was a relapse of the disease (text-fig. 5). If it can be shown that these changes precede the alterations of the morphological elements in the blood and in the bone marrow, this fact will be useful in early recognition of the relapse of the disease.

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Continuously Propagating Cells Derived From Normal Mouse Bone Marrow [1,2]

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SUMMARY

Serums from various animals were tested as adjuvants to synthetic medium to determine the most effective menstruum for growth of cells from explanted normal mouse bone marrow. Fetal calf serum was particularly effective in bringing about prolonged growth. The technique of tantalum-wire overlay of the explants and the use of fetal calf serum have enabled us to obtain large numbers of continuous cell lines. The histories and description of several of these lines are given. A medium deficient in glucose and high serum levels favored development of monocytic-like cell lines, whereas in the glucose-supplemented medium a large, flat-cell type appeared to have the selective advantage. These lines were shown through cytological studies not to be contaminants from established cell lines. The one heteroploid cell line tested gave negative results for possible protective action in recipient irradiated isologous mice.—J. Nat. Cancer Inst. 25: 1127–1139, 1960.

IN PREVIOUS reports the effectiveness of tissue-cultured mouse bone marrow in protecting recipient, irradiated, isologous mice was shown to be short-lived (*1*, *2*). One difficulty was that a large and continuous fall in the number of cells *in vitro* occurred during the first several days of culture due primarily to cell maturation and subsequent degeneration of such cells. Few cells remained for assay after 1 to 2 weeks in culture. This report describes the development of environmental conditions leading to almost 100 percent efficiency in obtaining continuously culturable cell lines from bone marrow of normal mice. In addition, the morphological characteristics of such cultures and their potential as hemic cells have been studied and are described. To the large list of human hemic cell lines developed (*3*, *4*) can now be added a number of murine bone marrow derived lines.

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[4] The authors wish to express their appreciation to Mr. J. D. Carnes for his aid in preparing the photographs.

MATERIALS AND METHODS

Bone marrow procurement and culture.—The details of obtaining and explanting bone marrow from femurs of mice have been described (*5*). The medium finally used in these studies consisted of 80 percent chemically defined medium NCTC-109 supplemented with 20 percent fetal calf serum (Microbiological Associates, Bethesda, Md., and Colorado Serum Co.) plus 50 μg. of streptomycin and 80 units of penicillin per ml. In some experiments McCoy's medium 5a (*6*) minus glucose was used. To insure sterility, the complete medium was filtered through HA-type Millipore filters as part of the routine of preparation.

The feeding schedule used in most of these studies was replacement of one half to two thirds of the old medium with fresh medium every 7 days.

Preparation of subcultures.—To harvest the cells for subculture, a 0.25 percent solution of trypsin in Hanks' salt solution lacking calcium and magnesium salts was used. The trypsin was added to the containers after complete removal of the medium. After the containers were prewarmed in a water bath at 37° C., incubation at 37° C. for 5 to 30 minutes, dependent on individual strains, was followed by agitation of the cells with a pipette. This latter step decreased the time needed to dislodge the cells from the glass surface and thus avoided prolonged exposure to trypsin. These cells were diluted in cold medium and harvested as soon as possible in a Servall refrigerated centrifuge at 121 × g for 12 minutes. The pellet was resuspended into fresh medium for inoculation into appropriate containers.

Strains of mice used and X-ray dose.—C3H or (C3H × 101)F_1 mice were used as bone marrow donors. In the "protection" experiments isologous mice, 3 to 4 months of age, were used as irradiated recipients for testing the cell lines derived from normal bone marrow. The details of exposure and method of injection have been reported (*1*).

RESULTS

Origin of the Houston and Anderson Bone Marrow Cell Strains

Nutritional aspects.—In the earlier studies on tissue culture of mouse bone marrow, we had found that an 80 percent medium 199 plus 20 percent horse-serum medium would allow maintenance of cells, as well as some division, for 1 or 2 months (*2*, *5*). In such a medium a few cultures would persist for as long as 3 or 4 months but showed continued regression until death of all cells had occurred. A study of the effect of combining NCTC-109 medium with horse serum on bone marrow cultures indicated this synthetic medium was superior for our purposes. A number of other serums were tried with this synthetic medium, as illustrated in table 1. Only calf serum seemed to be equal to horse serum for obtaining growth during initial culture. When fetal calf serum became commercially avail-

able, it was tried as an adjuvant (20%) to NCTC 109 synthetic medium. It was immediately apparent that fetal calf serum was superior to any other serum studied. In the use of such a medium in conjunction with our tantalum-wire overlay technique, we found that bone marrow derived cells could be cultured indefinitely, if there were no unforeseen accidents, such as leaky seals, microbial contamination, or overheated incubators.

TABLE 1.—Influence of serum source on C3H mouse bone marrow tissue cultivation

Serum added	Final concentration (*percent*)	Appearance of culture*	
		7 Days	30 Days
A. None		−	−
B. Isologous			
	10	−	−
	20	−	−
	10†	−	−
	20‡	−	−
C. Homologous			
C57BL	20	±	−
A	20	−	−
D. Heterologous			
Horse	5	+	±
	10	+++	++
	20	++++	++++
Calf	10	++++	++++
Chicken	10	++	−
Rabbit	10	−	−

*++++ = Healthy culture; − indicates complete degeneration.
†Incubated at 56° C. for 1 hour prior to use.
‡Ascitic fluid.

A study of the effects of feeding, by observing *p*H changes (phenol red indicator from orange-red to yellow) or a routine schedule independent of *p*H change, indicated little gross difference in culture characteristics after either procedure.

Histories of the cell lines.—The established cell lines are listed in table 2. The Houston lines (H) were obtained from pooled bone marrow explants obtained from the femurs of 3- to 6-month-old male or female C3H mice. We currently have under development the Anderson lines (A) that repre-

TABLE 2.—Histories of cell lines

Cell line	Date of original explants	Medium composition	Date of first subculture	Number of subcultures to 6/1/60
H-1a	1/27/59	NCTC 109 plus 20 percent fetal calf serum	5/6/59	21
H-1b	1/27/59	"	9/28/59	13
H-1e	1/27/59	"	11/3/59	11
H-4a	8/13/59	McCoy's 5a (minus glucose) plus 40 percent fetal calf serum	10/21/59	8
H-4b	8/13/59	"	10/21/59	9
H-4c	8/13/59	"	10/21/59	11
H-4d	8/13/59	"	1/29/60	1

sent cells derived from bone marrow of (C3H × 101)F_1 mice. The A lines have only recently been subcultured and are not described.

Culture Development and Morphology

Morphogenesis of the cell lines.—Hemic cells of the myeloid or erythroid series could be easily identified in our bone marrow cultures for 1 to 2 weeks, though the number and types of cells showed a continuous decrease. We have previously reported that cells from such 1- to 2-week cultures afford protection, decreasing with increasing time *in vitro*, to recipient lethally irradiated isologous mice (*1*, *2*). Invariably, a large, flat, mononuclear cell (Ep-L? of Berman and Stulberg) became the predominant species. In several hundreds of developing cultures, these mononuclear cells, or their progenitor, were always first observed singly or in small clumps by the 3d to 4th day of culture (fig. 1). These continued to proliferate (fig. 2) until one group of cells became confluent with its neighbors. The cells were attached to each other firmly enough so that disturbing an area of intensive growth, as was done by removing or agitating the wire screen, caused breaks in the monolayer and subsequent contraction into areas of heavy concentration.

The tantalum wire is important in the primary culture. Besides serving to hold the bone marrow fragments in place, the wire seems to favor development of the rapidly dividing cells. After 2 to 4 weeks of culture, some cell attachment and growth were seen on the wire web forming sinus-like structures. The growth became heavy in subsequent culture until the wire squares were completely covered in some areas (fig. 3). When such screens were removed from the container and the attached cells subcultured without further treatment by insertion into a T-flask, mononuclear cells grew on the glass surface within a few days. Thus the cells, resembling fibroblasts on the screen support, appeared to assume an epithelial-like form upon attachment to the glass. In most experiments the primary culture was trypsinized and the free cells and screen were transferred together in the first subculture.

In most cultures the predominant cell type at the time of initial subculture was a large, epithelial-like cell (fig. 4). Stained preparations usually revealed a rather pleomorphic morphology (figs. 5 and 6). There were several nucleoli in the large nucleus which were easily visible in the living cells and in properly prepared stained preparations.

The other cell type surviving at time of subculture was a slower growing, monocytic cell (fibroblast?) that in most cultures appeared separate and not connected with its neighbor (fig. 2). These monocytes could be completely overgrown by the more rapidly growing epithelial-like cells and were revealed when the sheet of covering cells was disturbed. These monocytes usually disappeared upon continued subculture.

In addition, it appeared that the use of NCTC 109 plus fetal calf serum favored growth of the epithelial-like cell type. It was in this medium that the H-1 series of cell lines were developed. By using a glucose-deficient

McCoy's 5a medium plus 40 percent fetal calf serum, which apparently was more favorable for monocytic growth, we established a propagating culture consisting primarily of monocytic-like cells (H-4c), described in table 2 and illustrated in figures 7 and 8. Initial chromosomal studies established that the cell stem line was diploid in number, but subsequent investigation has shown a tendency to polyploidy (*7*). Several other cell lines have also been established in this medium and retained for further studies (*see* table 2 and figs. 9 and 10).

Protective capacity of cell lines.—Injection of H-1a cells into lethally irradiated isologous mice did not alter the course of radiation-induced death (table 3). Only limited numbers of these cells could be injected intravenously without causing immediate death of the recipient. This may be due to their large size or the presence of small clumps of cells. Injection of more than 2×10^6 cells usually proved lethal. Washing the cells after harvesting from the trypsin-containing fluid did not negate this effect.

TABLE 3.—Effects of cell-line injection into irradiated mice

Experiment number	Treatment of C3H recipients	Cell strain	Cell dose	Number surviving 30 days	
				Controls	Treated animals
121859	750 r*	H-1a	$7–8 \times 10^5$	0/7	0/4
122959	750 r	H-1a	2.2×10^6 1.1×10^6 0.22×10^6	3/10	0/1 0/4 0/1
22460	700 r	H-1b	3.2×10^6 1.5×10^6 1.0×10^6	0/3	0/1 0/4 0/3
Total				3/20	0/18

*X-ray exposure from a General Electric Maxitron 250; radiation factors were 250 kvp, 30 ma., 1 mm. of aluminum added filtration, dose rate in air approximately 85 r per minute.

DISCUSSION

Reports of development of continuously cultured cells derived from normal mouse bone marrow have appeared in the literature (*8*). We found that several of these cell lines lacked protective activity when injected into irradiated isologous or homologous mice (*5*). A subsequent report from the laboratory in which the cell lines originated indicated that these lines were not derived from hemic tissue but were contaminants of established cell lines originally obtained from tissue other than bone marrow (*9*). Cytological studies on our mouse bone marrow lines indicated that they were not contaminated with any known strains, either human or mouse (*7*). The nature of the original stem cells in the bone marrow from which our cell lines have been derived is unknown. Bone marrow as

obtained here contains stromal as well as hemic cell types. For example, capillary fragments were found in the explanted bone marrow fragments. It is not possible at this time to determine whether the cell lines were obtained from cells with full hematopoietic potential or from the stroma. If the lines were derived from the latter type of cell, it would be expected that they would not exhibit a capacity to protect recipient irradiated mice. Another complicating factor in drawing conclusions concerning the lack of protection by the cell lines tested was their heteroploid nature (*7*). It is not unreasonable to expect that even if such cells were derived from true hemic types they were so altered in genomic make-up that much of their hemic potential had been lost or altered. Furthermore, because of the nature of the difficulty in injecting large numbers of these cells intravenously, both the dose of cells injected and the number of animals tested were less than desired. Our results may only be considered as suggestive that such cell lines do not protect recipient animals. It has been shown that 1×10^6 fresh bone marrow cells will protect the majority of irradiated recipient, isologous (C3H $\times$ 101)F_1 mice (*10*).

Efforts to prevent ploidy changes through nutritional control may prove useful in maintaining functional cells. In our present experiments, the use of a synthetic medium lacking in glucose has provided us with a cell, the stem line of which initially was diploid in number (*7*). These cells are monocytic in character and not very different morphologically from monocytes observed in fresh culture. We hope to test such cells for protective activity.

The importance of nutrition in providing the necessary conditions for development of cell lines was obvious in our studies. After tests of many nutrients in which all attempts to obtain cell lines failed, it was the addition of fetal calf serum that provided the necessary factors for allowing a portion of the cells to adjust to the new environment. Although not subject to objective testing, fetal calf serum appeared to contain necessary factors not found in calf serum. Even though fetal calf serum appears to be the superior adjuvant for development of cell lines, preliminary tests on the maintenance of protective cells in "nontransformed," short-term cultures have shown that in this respect it is only slightly superior to horse-serum supplements.

Detailed chromosomal analysis of some of these lines will be reported in a subsequent publication.

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PLATES

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PLATE 148

Photomicrographs showing features of primary bone marrow culture.

FIGURE 1.—Bone marrow culture at 4 days. × 450

FIGURE 2.—Bone marrow culture at 41 days, showing character of growth of rapidly growing, flat cells on the glass surface. *Note* individual monocytes in upper portion of field. × 45

FIGURE 3.—Bone marrow culture at 47 days. Photograph of growth on tantalum-wire screen used in primary culture. *Note* fibrous growth and the formation of sinus-like openings as the elongated cells grow across wire square. Several of these wire squares have been completely covered in this culture. × 29

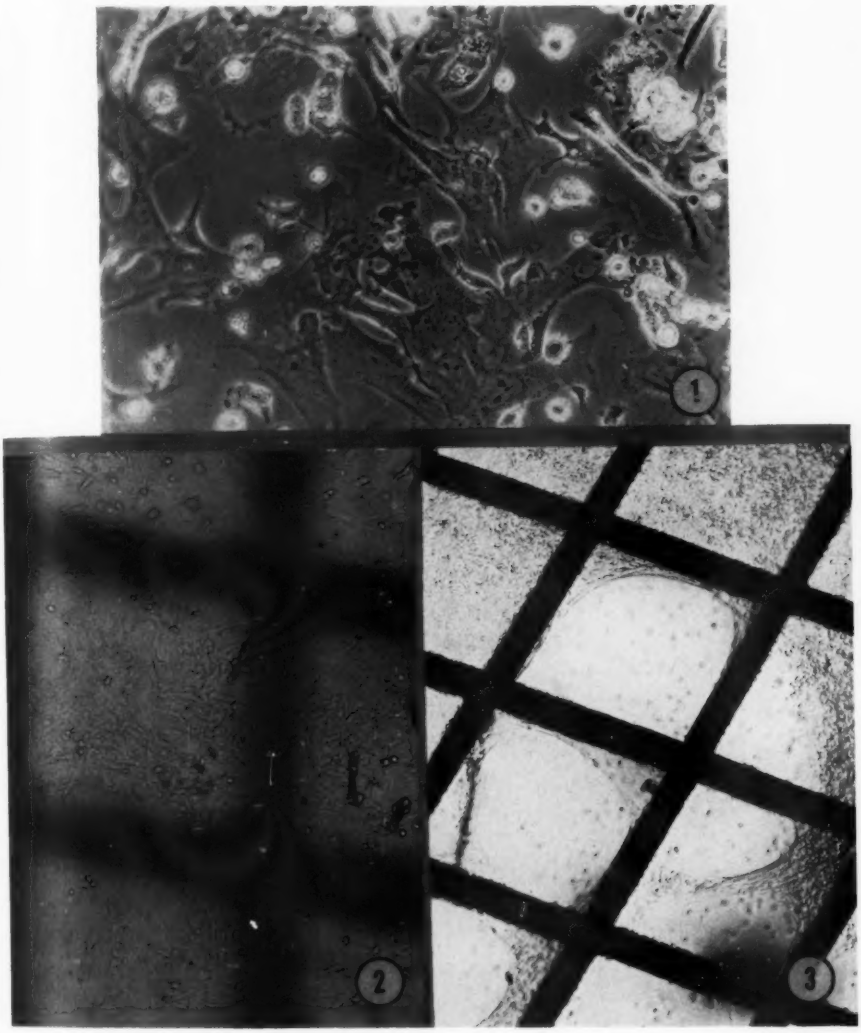

1
2
3

PLATE 149

Photomicrographs showing cultural characteristics and morphology of H-1 cell lines.

FIGURE 4.—H-1a line growing on flying coverslip. *Note* confluent growth of cells also prominent nucleoli. × 450

FIGURE 5.—Giemsa-Wright's stained preparation of H-1a line, 20th subculture. *Note* large size and pleomorphic character. × 1800

FIGURE 6.—Giemsa-Wright's stained preparation of H-1b line, 13th subculture. × 1800

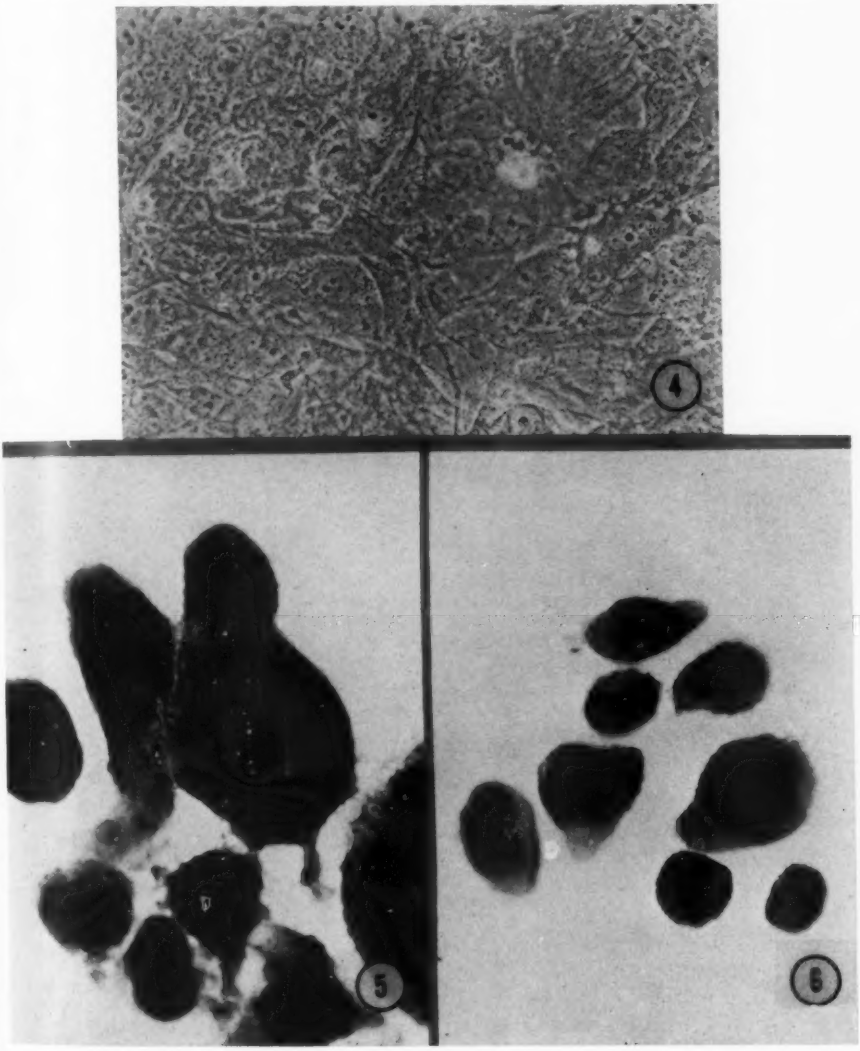

4
5
6

PLATE 150

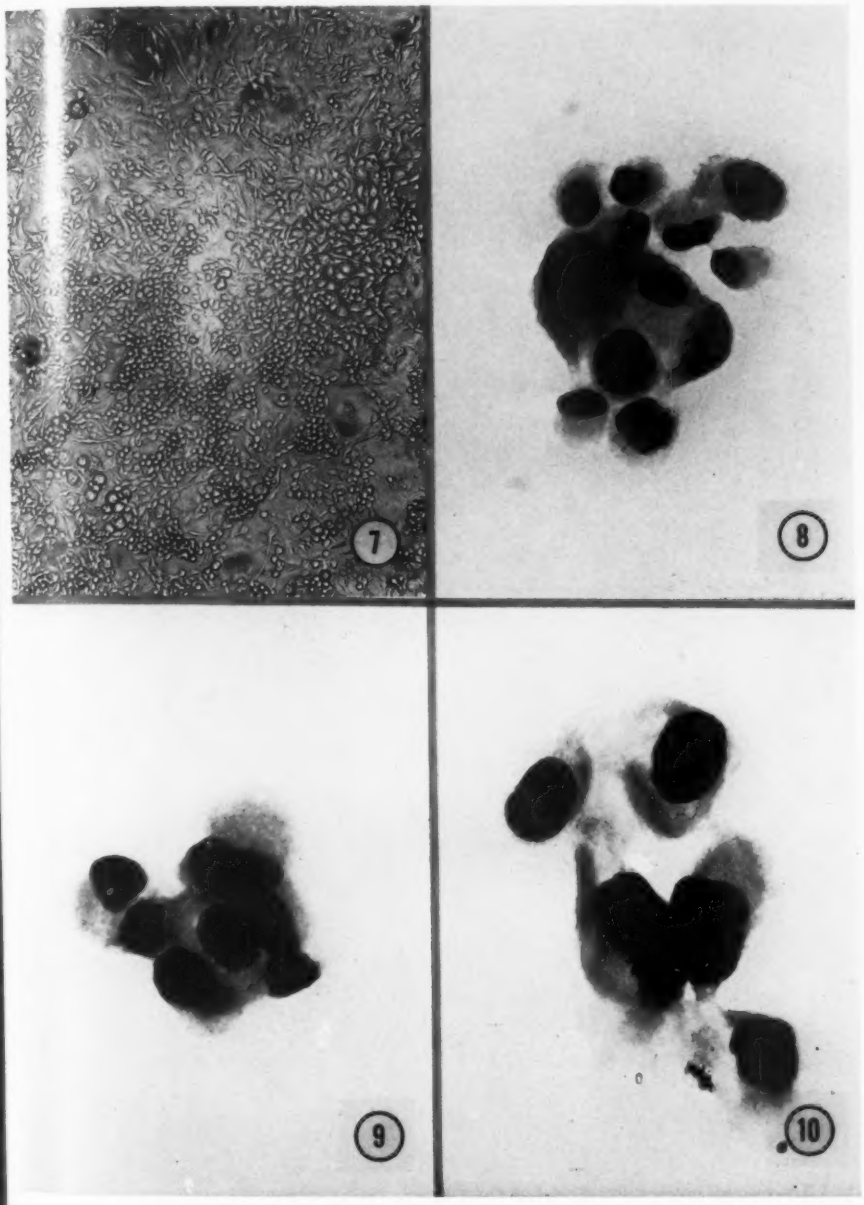
Photomicrographs showing cultural characteristics of H-4 cell lines.

FIGURE 7.—H-4c line growing on surface of T-flask. *Note* monocytic-like appearance of the majority of cells—7th subculture. × 45

FIGURE 8.—H-4c line, 8th subculture, Giemsa-Wright's stain. Small cells resemble monocytic cells of primary cultures. × 1,800

FIGURE 9.—H-4b line, 8th subculture, Giemsa-Wright's stain. × 1,800

FIGURE 10.—H-4a line, 8th subculture, Giemsa-Wright's stain. *Note* large size of cells as compared with small cells in H-4c. × 1,800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10

Endometrial Sarcomas of the Uterus and Carcinosarcomas of the Submaxillary Salivary Gland in Castrated A × C Strain Female Rats Receiving *N,N*-Fluorenyldiacetamide and Norethandrolone [1,2,3]

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SUMMARY

In 10 castrated female A × C rats receiving *N,N*-fluorenyldiacetamide (2FdiAA) and norethandrolone, 3 developed sarcomas of the uterus and 2 carcinosarcomas of the salivary glands. It appears that norethandrolone causes endometrial cystic hyperplasia which with 2-FdiAA leads to endometrial sarcomas of the uterus. The carcinosarcomas of the salivary gland did not develop in intact animals of either sex and may be related to the anabolic effects of norethandrolone. Salivary-gland tumors did not occur in similarly treated castrated male rats with or without norethandrolone. Neither tumors of the uterus nor salivary glands were observed in intact females given norethandrolone or in intact or castrated female rats given progesterone or testosterone. Hence, both the absence of the gonads and the presence of norethandrolone seem to be important in the induction of uterine sarcomas or salivary-gland carcinosarcomas in A × C rats fed 2-FdiAA.—J. Nat. Cancer Inst. 25: 1141–1153, 1960.

N-2-FLUORENYLACETAMIDE (2-FAA) and *N,N*-fluorenyldiacetamide (2-FdiAA) have produced tumors which vary in distribution, depending on the dosage and strain of rat (*1–3*). Tumors have been seen most often in the liver, mammary gland, and ear canal, but they have also been observed in the uterus (*4*) and the salivary gland (*5*). 2-FAA, when given to animals with hyperplasia of the thyroid gland secondary to allyl-thiourea, will result in carcinomas of the thyroid (*6*). Therefore Cantarow, Paschkis, Stasney, and Rothenberg gave sex hormones to rats to determine whether the hyperplasia of the uterus, ovaries, prostate, seminal vesicles, and testes would, in the presence of 2-FAA, lead to the development of neoplasms in these organs (*7*, *8*). They were unable to produce tumors of the target organs.

In previous work in this laboratory on liver carcinogenesis with 2-FdiAA, A × C strain rats, and hormones, neither uterine nor salivary-gland tumors have been seen in intact or castrated female rats or similar animals

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[2] Aided by a grant from the American Cancer Society, Inc.

[3] This research was started at the University of Kansas and completed at the University of Maryland, the present address of the author.

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receiving carcinogen and large doses of progesterone or testosterone (*9*). There have been tumors of these organs in castrated female rats receiving carcinogen plus norethandrolone (17α-ethyl-19 nortestosterone).[4] The administration of norethandrolone was not associated with salivary-gland tumors in intact females, males or castrated males, or with uterine tumors in intact females. One small, similar salivary-gland tumor has been found in one castrated male receiving testosterone and 2-FdiAA. Spontaneous uterine and salivary-gland tumors have not been noted in about 2,000 rats of this strain over the past few years.

MATERIALS AND METHODS

Groups of 15 intact and castrated male and female A $\times$ C rats, weighing 175 to 225 gm. and 3 to 4 months of age, were fed the Morris Diet #272.[5] The diet consisted of 300 gm. commercial casein, 2275 gm. skim-milk powder, 6152 gm. ground hard spring wheat, 200 gm. Brewer's yeast, 200 gm. desiccated whole-liver powder (Wilson), 140 gm. sodium chloride, 10 gm. ascorbic acid, 13 gm. ferric citrate, 100 gm. cod-liver oil, 610 gm. corn oil, and 0.025 percent 2-FdiAA. They received the carcinogenic diet for 4 weeks, alternated with 1 week of basal diet, until the carcinogen had been administered for a total of 4 months. Thereafter they were continued on the basal diet for 5 months, at which time they were killed. Similar groups of intact and castrated male and female animals received norethandrolone, testosterone propionate, or progesterone. A dose of 1.0 mg. norethandrolone in 0.1 cc. of saline was injected subcutaneously every 3 days throughout the experiment. Testosterone and progesterone pellets, which consisted of 1 part hormone and 3 parts cholesterol, were implanted subcutaneously in a dose of 20 mg. per 100 gm. of body weight. All hormones were administered from the time the carcinogen was started in the diet.

The rats were killed by exposure to ether inhalation. When tumors of the salivary gland were observed at autopsy, they were transplanted subcutaneously into the groin of 3 to 5 A $\times$ C male or female rats and 5 Wistar male rats. Transplants that grew were carried serially for 5 generations. Tissues were fixed in 10 percent formalin, sectioned, and stained with hematoxylin and eosin. The tumors of the uterus and salivary gland were also stained with Masson's trichrome, Gridley's reticulum, phosphotungstic acid hematoxylin, and periodic acid-Schiff (PAS). The pituitary glands were stained with PAS-orange G.

RESULTS

Ten castrated female animals survived between 25 and 40 weeks, *i.e.*, long enough to develop tumors of the liver. Uterine tumors were ob-

[4] Kindly supplied by Searle and Company, Chicago, Ill., as Nilevar.
[5] Personal communication: H. P. Morris, National Cancer Institute, Bethesda, Md.

served in 3 rats, salivary-gland tumors in 2, and hepatomas in 6. The uterine and salivary-gland tumors were not in the same animals. The 2 rats with salivary-gland tumors also had hepatomas; however, in the 3 rats with uterine tumors there were no tumors of the liver. There were no uterine or salivary-gland tumors in intact females, but 9 of 13 intact female animals developed hepatomas.

One of 10 castrated male rats receiving testosterone developed a small carcinosarcoma of the salivary gland. The remaining groups of animals did not have salivary-gland or uterine tumors.

The tumors of the liver will be reported in another paper (*9*).

Uterine Tumors

The tumors of the uterus were all grossly similar. They were soft, purple-gray, measured as much as 3.0 × 3.5 × 6.6 cm., and replaced large areas of the uterus. In 2 of the 3 animals there were peritoneal implants of tumor. In 2 animals there was a small amount of peritoneal bloody fluid; in the third, in addition to the peritoneal tumors, the abdomen was distended by bloody fluid and blood was oozing from the vagina. The cut surface of each tumor was soft, gray, and fleshy, and there were areas of hemorrhage and necrosis (fig. 3). Microscopically, the tumors were made up of cells that varied somewhat in their differentiation. The better-differentiated cells were spindle-shaped with eosinophilic cytoplasm and tended to form bundles. The nuclei were often large, round or oval, vesicular, and hyperchromatic, with infrequent nucleoli or mitoses. There were no cross striations or myofibrils in the tumor cells. By contrast, the anaplastic areas were present as rather solid cellular growths of individual cells. The cells had nuclei that were generally small to medium-sized, round or oval, hyperchromatic, at times vesicular with a prominent central nucleolus and generally rather scanty basophilic cytoplasm. Occasional cells were large with several nuclei and nucleoli. Mitoses were common.

There were foci of necrosis in the tumor and the stromal content varied. One tumor had moderate connective-tissue stroma (fig. 1), one had little stroma, and the other had fine collagen and reticulum comparable to that seen in the normal endometrial stroma (fig. 5). Tumor cells in all instances involved the endometrium and invaded the smooth muscle of the wall of the uterus. It is likely that all 3 were endometrial sarcomas, but the possibility that the tumor with abundant collagenous stroma might be a leiomyosarcoma cannot be entirely discarded. Sections of the peritoneal implants were entirely similar to the primary uterine growth.

In the animals with tumors of the endometrium there was accompanying cystic hyperplasia (fig. 4). The endometrial glands were increased in number and were lined by tall cells with clear cytoplasm and basal nuclei. The cystic glands were lined by flattened cells and contained mucus. In the dense stroma there were prominent venules and in some animals

decidual cells. Occasionally, red blood cells were noted within the stroma and the lumens. The endometrium of the uteri of the remaining castrated animals receiving norethandrolone was mild to moderately hyperplastic with cysts. In the intact females receiving norethandrolone there were corpora lutea and follicles in the ovaries and the endometrium was not unusual.

The sarcomas of the endometrium were not transplanted.

Salivary-Gland Tumors

These tumors measured up to 2.5 × 3.0 × 3.1 cm. and were hard and encapsulated, except where they had invaded skeletal muscle and bone of the jaw (fig. 2). Grossly, they appeared to have completely replaced the gland. The cut surface was firm, pale, and fleshy. Histologically, the tumors were similar and composed of 2 types of cells, epithelial and stromal. Much of each tumor was composed of spindle-shaped cells, at times in bundles, spreading in several directions. The nuclei were large, vesicular, and hyperchromatic, oval or round, with 1 or 2 prominent nucleoli. The cytoplasm was generally abundant, finely granular, and palely basophilic or eosinophilic. Areas of these cells merged into and mixed with undifferentiated cells with similar nuclei but with scanty cytoplasm. The intercellular stroma varied from scanty to abundant and consisted of collagen and fine connective-tissue fibers, often with rich, delicate reticulum fibers. In a few areas myxoid tissue or foci of hyaline matrix were seen. There were scattered tumor giant cells with 2 or 3 nuclei. Mitotic figures were common and often atypical. No cross striations were observed.

The epithelial component was composed of polygonal cells with dark, eosinophilic or at times basophilic cytoplasm and large, hyperchromatic, oval nuclei. The epithelial cells were often arranged in small sheets or islands, but in some areas they were interspersed with spindle-shaped or undifferentiated cells (fig. 7); in other areas there was spindling of epithelial cells with invasion of the stroma simulating a sarcomatous transformation of the epithelial component. Around the edges and within the periphery of the tumor were small acini and ducts. The acini were lined by a single layer of cuboidal cells representing fragments of salivary gland and composed of cells with small, dark nuclei and basophilic cytoplasm. The ducts were lined by a single layer of low cuboidal cells with eosinophilic cytoplasm (fig. 6). The acini sometimes contained a small amount of homogeneous eosinophilic material, and the lining cells contained a small amount of granular diastase-resistant PAS-positive material in the cytoplasm. Small amounts of diastase-resistant PAS-positive material occasionally were also seen in small ducts.

On the edges there were normal parotid gland, sublingual gland, skeletal muscle, small nerves, and lymphoid tissue, all of which in places were invaded by tumor cells. But uniformly the gland chiefly involved was the submaxillary gland. Frequently there were dilatation and distortion

of the ducts and atrophy or disappearance of the acini of the trapped glands. In other areas, tumor cells appeared to be arising from the duct walls and invading the adjacent stroma.

The salivary glands in 2 of the other 8 rats showed minor changes. In one there was slight dilatation of the ducts of the parotid gland; in the other there were slight cystic change and proliferation of the ducts of the submaxillary gland.

Transplantation Results

The carcinosarcomas of the salivary gland grew rapidly in the A × C strain rats and were carried for 5 generations. After 2 months' growth, the average animal weighed 190 gm., exclusive of the tumor; the average tumor weighed 140 gm., and its size varied from 8 to 12 cm. in the greatest diameter. Ulceration was often seen in the overlying skin. The tumors were cystic, with approximately 1 cm. of viable tumor around the periphery and 0.5 cm. of necrotic tissue surrounding a central cavity containing light-yellow fluid. Microscopic study revealed that the transplants were sarcomatous and resembled the original sarcomatous portion of the primary tumors except for an increase in tumor giant cells. There was invasion of skeletal muscle of the abdominal wall. Small metastases were in the lungs of 10 of the 20 host animals. Transplants to Wistar rats did not survive.

Other Organs

The pituitary glands in the castrated rats consisted of a mixture of chromophobic and acidophilic cells, with rare basophilic cells containing small, clear vacuoles and with rare, sparsely granulated basophils. In the intact animals sparsely granulated basophils were more conspicuous. The lungs were not involved by metastases from the uterine or salivary-gland tumors. Tumors of the liver in all instances were well-developed and differentiated hepatocellular carcinomas. Three of the castrated females had metastases of hepatocellular carcinoma in the lungs and one intact female had an embolus of similar tumor in the lung.

DISCUSSION

Castrated A × C female rats receiving 2-FdiAA and norethandrolone developed sarcomas of the uterus and carcinosarcomas of the salivary gland. Intact females and intact or castrated males similarly treated did not develop sarcomas, nor were there sarcomas in female rats or intact or castrated females given testosterone or progesterone.

Although norethandrolone has mild androgenic and progestational effects, it is predominantly anabolic (*10–13*). Not only is its estrogenic effect negligible (*14*), but it is even somewhat antiestrogenic (*15*). It inhibits gonadotrophin secretion by the pituitary (*16*). The latter was supported by the absence of castration cells and the rare basophils in the pituitary glands in the castrated animals given norethandrolone.

The castrated female rats that received norethandrolone had cystic glandular hyperplasia and stromal hyperplasia of the endometrium, which was more marked in the animals with tumors. It was observed, therefore, that 2-FdiAA in the presence of endometrial stromal hyperplasia led to the development of endometrial sarcomas, but that the hyperplasia of the glandular epithelium did not lead to neoplasia of the glands. Intact females receiving norethandrolone and animals given testosterone or progesterone did not develop such endometrial hyperplasia and they did not show tumors. It is noteworthy that Ducommun *et al.* (*17*) previously reported endometrial hyperplasia in intact Wistar rats with norethandrolone dosages more than 3 times those used in this experiment.

Marked stromal hyperplasia induced by estrogen might be expected to respond to 2-FdiAA such as in hyperplasia induced by norethandrolone, but intact rats receiving estrogen and 2-FAA in the experiments of Paschkis *et al.* did not develop tumors of the uterus (*7*, *8*). The majority of the animals developed pyometra, which may have influenced the results. No one to our knowledge has reported studies on carcinogen-treated castrated female rats given estrogen.

Uterine fibrosarcomas have been seen with notable frequency only in Buffalo-strain female rats (6 of 52) fed 2-FAA (*4*). Four other strains including A × C rats did not develop uterine tumors. It is interesting that there was an inverse relationship between the incidence of mammary-gland tumors and tumors of the liver. In the present study the rats with uterine tumors did not have tumors of the liver, while 6 out of 7 of the animals without uterine tumors had hepatocellular carcinomas. The tumors of the uterus or liver were observed at approximately the same time after the start of the experiment, which suggests that the occurrence of one tumor did not mask the appearance of the other. It is apparent that the rats that responded with marked endometrial hyperplasia developed uterine sarcomas and those with little or only moderate hyperplasia did not develop sarcomas.

Mixed tumors are the most common tumors of the salivary gland in man. Attempts to produce mixed tumors of the salivary glands in animals have been notably unrewarding. The tumors of the salivary gland induced in rats with 2-FAA by Heiman and Meisel were adenocarcinomas (*5*). Carcinogenic pellets have been placed in the salivary glands of animals and have produced a variety of tumors, the most common of which has been squamous-cell carcinoma (*18–20*). Steiner did obtain a few mixed tumor types containing carcinosarcoma in rats by using pellets of benzopyrene (*20*). Opinion has differed as to whether the source of the tumors was limited to the intercalated ducts (*18*, *19*), or whether the acinar cells were also neoplastic (*20*).

In the present experiments carcinosarcomas of the salivary gland occurred in castrated female rats given norethandrolone. The only other animal that developed a small carcinosarcoma of the salivary gland was 1 of 10 castrated male rats receiving testosterone. The tumor in this rat was not apparent until the animal was killed. The absence of

these tumors in intact animals of either sex, or castrated females treated with hormones other than norethandrolone, suggests a protective effect of the gonads and might implicate the anabolic rather than the progestational effect of norethandrolone. The only changes in intact male rats or castrated female or male rats receiving 2-FdiAA, but not treated with hormones, were occasional slight cystic proliferation of the ducts of the salivary gland, but no distinct early neoplastic changes were evident.

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PLATES

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PLATE 151

FIGURE 1.—Endometrial sarcoma seen in figure 3, showing abundant areas with collagenous stroma. Masson. × 217

FIGURE 2.—External surface of 2.5 × 2.7 × 3.0 cm. firm, pale, fleshy carcinosarcoma of the submaxillary gland with invasion of muscle and jaw.

FIGURE 3.—Cut surface of soft purple-gray 2.5 × 3.0 × 4.5 cm. endometrial sarcoma with necrotic yellow center. There were 5 mm. purple peritoneal implants and bloody peritoneal fluid (not shown).

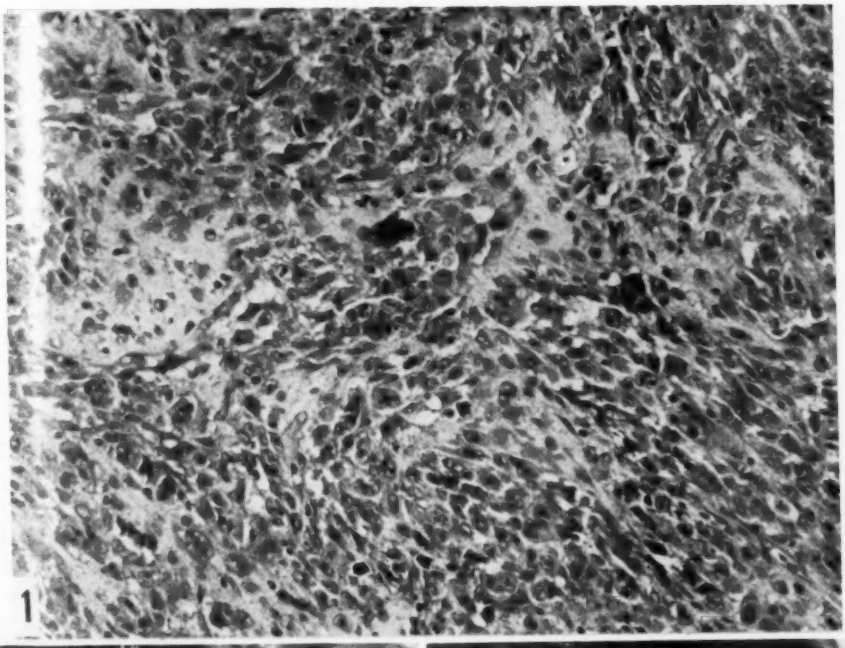
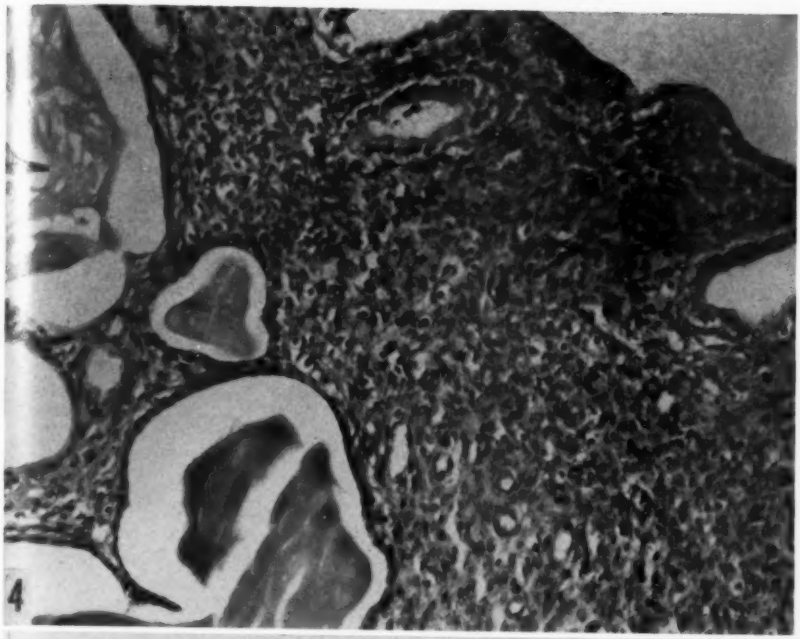


PLATE 152

FIGURE 4.—Cystic hyperplasia of endometrium taken from area of uterus not involved by tumor in same animal as figure 5. Hematoxylin and eosin. × 207

FIGURE 5.—Another endometrial sarcoma replacing endometrium in which tumor cells resemble those of endometrial stroma. Hematoxylin and eosin. × 415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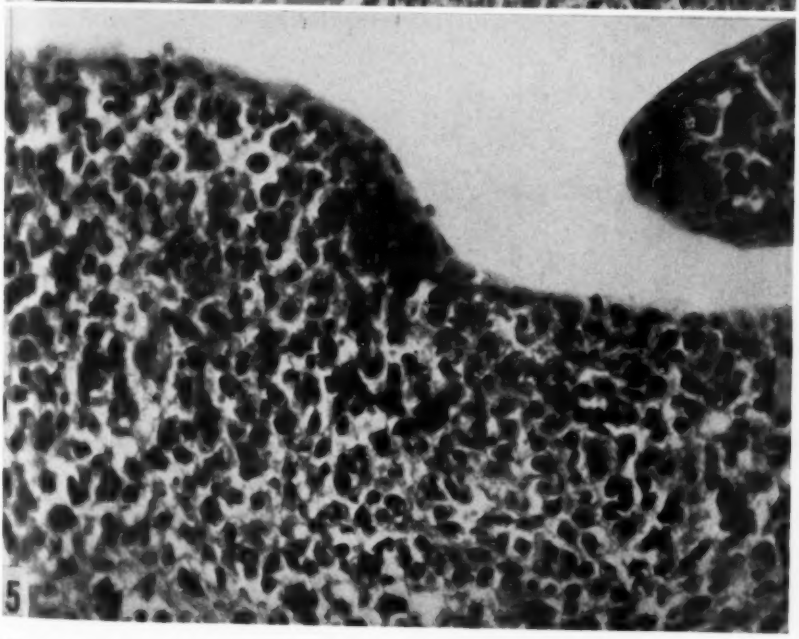
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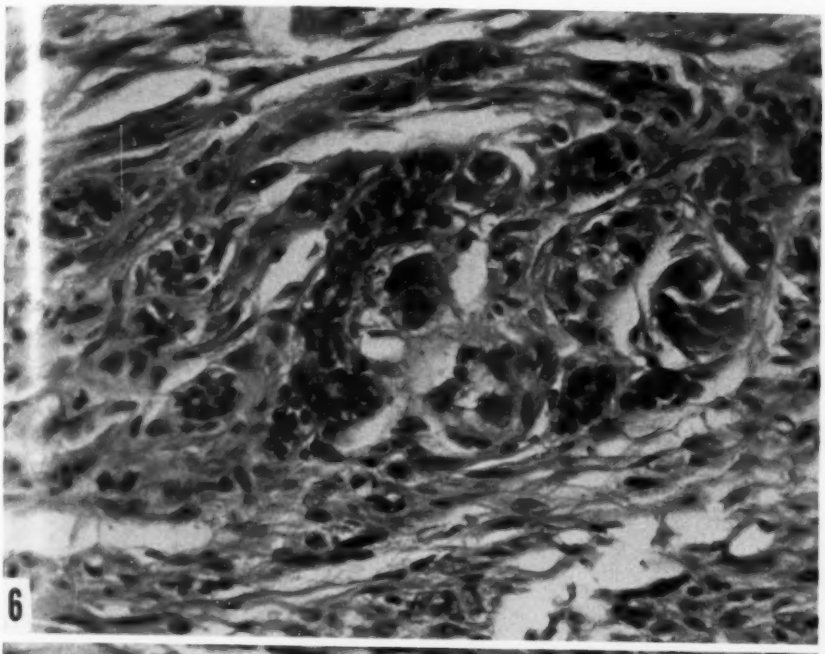
PLATE 153

FIGURE 6.—There are islands of ducts that are trapped and compressed by carcinosarcoma of the submaxillary salivary gland. Hematoxylin and eosin. × 415

FIGURE 7.—Sarcoma with nest of carcinoma from animal shown in figure 2. Hematoxylin and eosin. × 415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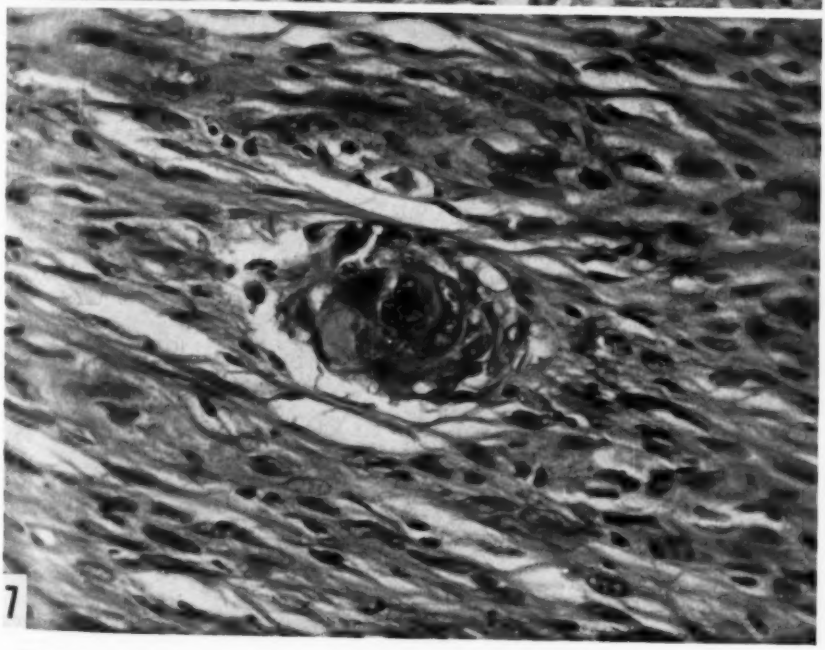
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Mechanism of Experimental Tumorigenesis. VI. Ultrastructural Alterations in Mouse Epidermis Caused by Locally Applied Carcinogen and Dipole-Type Tumor Promoter [1,2]

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SUMMARY

An electron microscopic study of the effect of locally applied 7,12-dimethylbenz[*a*]anthracene (DMBA) and 3-methylcholanthrene (MCA) on the interfollicular epidermis (IFE) of the back of the mouse was made. The pattern appeared to agree with that seen with the light microscope. Thus in light and electron microscopy, the hyperplastic response of the IFE that was caused by DMBA and MCA was totally different from and opposite to that of a locally applied dipole-type tumor promoter. Characteristic effects were: (*a*) abnormalities in cell surface-to-cell surface contact in which combined light and electron microscopy indicate disturbances in detachment of epidermal cells; (*b*) development of new patterns in the cytoplasm of nucleated cells; (*c*) accumulation of electron-dense inclusion bodies within mitochondria—the most characteristic and most regularly occurring change; (*d*) disturbances in keratinization; (*e*) an increase in nucleo/cytoplasmic ratio; and (*f*) variabilities in degree and quality of the changes in different places of the treatment area. When the skin was exposed to effective or prolonged doses of carcinogen, all these alterations were pronounced and the changes were easily reproducible. There was neither a reversion of the epidermal cells to normal basal cells nor to less-differentiated cell types; instead new cell types developed. Since related patterns were also evident in light microscopy on paraffin sections, the changes had arisen *intra vitam*. The nature and pattern of the alterations, by DMBA, by MCA, and by dipole-type tumor promoters, may clarify the regression mechanism of benign, regularly formed skin papillomas.—J. Nat. Cancer Inst. 25: 1155–1189, 1960.

EXPOSURE OF the skin of the back of the mouse to carcinogenic hydrocarbons results in numerous distinct changes, which can be seen with light microscopy in conventional paraffin-embedded sections. Except for hyperplasia, these changes in the epidermis are: (1) development of nuclear and cellular atypia and increase in the nucleo/cytoplasmic ratio, (2) development of and increase in compact basal cells, (3) formation of

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intercellular spaces and intercellular bridges between basal cells, (4) abnormalities of intercellular bridges and ribbon-like "sticky" connections between daughter cells, which indicate disturbances in and rearrangement of the epidermal elements, and (5) derangement in keratinization, and in mitotic activity in the pilosebaceous apparatus, and in the general organization. The changes vary in different places of the treatment area. The alterations are greater when the skin is exposed to prolonged or effective doses of carcinogen (*1*, *2*).

If related changes are observed in comparative skin elements and in the corresponding experimental series, and can be seen in light and electron microscopy in methacrylate-embedded sections, the alterations in the latter must be considered real and to have arisen *intra vitam* as a result of treatment with carcinogen. To show this is the purpose of the present study.

The ultrastructural characteristics of the epidermis of the adult mouse have been described (*3*). In light and electron microscopy, the epidermal alterations caused by Tween 60- or Span 60-type agents were comparable (*4*).

MATERIALS AND METHODS

The general techniques have been described previously (*1*, *3*). The carcinogens, 9,10-dimethyl-1,2-benzanthracene[5] (DMBA) and 20-methylcholanthrene[6] (MCA) (obtained from L. Light & Co., Colnbrook, England), were dissolved in reagent-grade acetone. The solutions were dropped with a glass pipette onto the back of the mouse.

With the two-stage technique, there was an interval of 30 days before posttreatment with technical (t.) Tween 60, Span 60, Tween 20 (Atlas Powder Co., Wilmington, Del.), laboratory-synthesized (s.) Tween 60 (polyoxyethylene sorbitan monostearate), and Span 60 (sorbitan monostearate) was begun (*1*, *4*, *5*).

Skin biopsies from the treatment area were taken on the 2d, 6th, 10th, 16th, and 30th day. Additional biopsies were taken at longer intervals—up to 343 days. Only interfollicular epidermis (IFE) of the back of the mouse was studied (*1*, *3*, *6–8*). Experiments with DMBA were repeated after an interval of 1 year.

The phase of the hair follicular cycle (anagen, catagen, or telogen) was determined as previously described (*6*, *7*). However in the experiments with carcinogens this was often impossible because of severe disturbances in the histologic architecture and damage of the pilosebaceous apparatus. Since the hair cycle spreads wavelike on the skin of the mouse, various growth phases can be observed simultaneously, even in the same histologic specimen.

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[5] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz [*a*]anthracene.
[6] *Chemical Abstracts'* nomenclature: 3-methylcholanthrene.

The following experiments were performed:

1. Continuous local treatment of the back of the mouse, 6 times a week: once daily, with 60 and 0.06 γ of DMBA and MCA.

2. A single initial local application of 320, 160, 80, 60, 40, 20, and 10 γ of MCA without posttreatment.

3. Continuous local treatment, 6 and/or 12 times a week: once and/or twice daily, with about 0.18 M aqueous solution of t. Tween 60 and s. Tween 60 after a single initial local application of 320 γ of MCA.

4. Continuous local treatment, 6 and/or 12 times a week, with about 0.18 M aqueous dispersion of t. Span 60 and s. Span 60 after a single initial local application of 320 γ of MCA.

5. Two histologically benign skin papillomas provoked with the two-stage technique, with t. Tween 60 and t. Tween 20 (polyoxyethylene sorbitan monolaurate) in posttreatment.

RESULTS

Electron Microscopy of Keratinizing Tissues

Physiologically characteristic of the keratinizing tissues is a gradual transformation of cellular proteins into keratogenous structures and, finally, into disintegrating, dead, horny material. Even the same section can contain patterns of highly varying mass and/or electron density. After conventional fixation, the ultrastructural features of the lower layers of keratinizing tissues are well preserved, but those of the upper layers are definitely poor. This is particularly evident in multilayered keratinizing tissues, *e.g.*, human epidermis, hair and feather follicles, and mouse and rat footpads (*9–11*). However, this occurrence does not depend solely on the multilayered structure. Though the IFE of the back of the adult mouse of the present strain is, in principle, one-layered, the stratum granulosum and stratum corneum also present the same difficulties (*3*).

In the treatment of the back of the mouse with Tween 60-type products [t. Tween 60, s. Tween 60, polyoxyethylene-1,4-sorbitan monostearate, polyoxyethylene-1,4-sorbitan distearate, polyoxyethylene-1,4-sorbitan tristearate, polyoxyethylene-1,4,3,6-dianhydrosorbitol monostearate (*4*)], there was apparently a high-degree preservation of the ultrastructural features of nucleated cells, despite a strong IFE hyperplasia. The keratinization occurred only in the most superficial layers.

Treatment of the skin of the mouse with Span 60-type products [t. Span 60, s. Span 60, 1,4-sorbitan monostearate, 1,4-sorbitan distearate, 1,4-sorbitan tristearate, and 1,4,3,6-dianhydrosorbitol distearate (*4*)] showed an accumulation of electron-dense material in the cytoplasm of the basal cells that is not seen in these cells in normal IFE. This electron-dense material is always seen in superficial layers of keratinizing tissues, and the more severe the alterations, the less preservation of ultrastructural features of the altered IFE.

Exposure of the skin of the mouse to DMBA and MCA caused disturbances in keratinization in all layers of the epidermis. These alterations could be verified by light microscopy (*1*, *6–8*), but satisfactory thin sections for electron microscopy were difficult to obtain.

On the other hand, the histology of the epidermis was comparatively well preserved when the skin was treated with Tween 60-type products after a single initial local exposure to DMBA and MCA. However, the degree of preservation essentially depended on the dose, of the carcinogen given in pretreatment—the larger the dose, the more severe the alterations and disturbances in keratinization. The electron microscopic observations presented should be considered against this background.

Experiment 1

Continuous Local Treatment (6 Times a Week) With 60 and 0.06 γ of DMBA and MCA

The general appearance under the electron microscope conformed to that seen under the light microscope (*1*). A progressive decrease in uniformity of the size and shape of the cells and nuclei was characteristic. Further, a concomitant derangement in general organization was evident. Allied to the alterations was the development of a disorderly epidermal hyperplasia. The severity of the changes was directly correlated to the length of exposure and the doses of DMBA and MCA: The alterations were more severe in the DMBA experiments; with 0.06 γ of DMBA and MCA the changes were sparse. The effect of carcinogens revealed a variety of patterns at different sites in the treatment area. Therefore a large amount of material and many preparations were needed. Ultrastructural changes were observed in cell surface-to-cell surface contact, cytoplasmic fiber formation, mitochondria, and in the stratum corneum. Figures 1 to 23 illustrate the effect of DMBA and MCA.

Cell surface-to-cell surface contact.—Nucleated cells in the IFE were separated from each other by wide and irregular *intercellular spaces*. This was apparent both in tangential or oblique sections (figs. 1, 4, and 9 to 11) and in sagittal sections (figs. 5 to 8, and 12). *Intercellular bridges* in many instances appeared irregular (figs. 1, 4, and 5). There was interdigitation and intermingling of cytoplasmic pseudopodia that formed an irregular meshwork in the spaces between nucleated cells. This was characteristic of the deranged IFE of all animals, irrespective of the length of treatment with carcinogen. Figures 5 to 7 illustrate connections between adjacent cells. Figure 5 presents a frequently observed pattern, where bundles of electron-dense fibrils and/or filaments almost fill the whole bridgehead. The same pattern is evident in figure 7 and, to some extent, in figure 11. This appearance was particularly evident between basal parental and daughter cells. Sometimes the large, irregular, and stretched intercellular bridges seemed to be arranged along the "lines of forces." In many places, even in vast areas, the intercellular bridges were lacking or ruptured [*cf.* text-fig. 1 in (*4*), a sketch illustrating the development of intercellular

spaces and intercellular bridges]. The alterations depicted are evident under the light microscope, and the more efficient the carcinogen treatment, the greater the changes. On the basis of light microscopic observations, this feature has been called "stickiness" between parental and daughter cells (*e.g.*, *2*). The ultrastructure of the *desmosomes* did not deviate from that seen in IFE of the normal mouse. The same was true of the *dermoepidermal junction* and *basal attachment devices* [figs. 4 to 6, and 13 to 15 in (*3*)]. However, material that histologically showed a distinct infiltrative, destructive mode of growth was excluded.

Cytoplasm.—In light microscopy on conventional paraffin sections, one of the most conspicuous changes arising as a result of continuous exposure to carcinogens was the development of and increase in relatively small, basophilic and "compact basal-type" cells (*1*, *6–8*). In electron microscopy, these cells deviated from the undifferentiated basal cells of the normal IFE (*3*) and from those in the Tween 60 experiments (*4*). Typical was the polymorphic image of the nucleated cells even in the same section. The *nucleo/cytoplasmic ratio* was, in general, increased, *e.g.*, figure 9. The *nucleo/cytoplasmic interface* was often scalloped, and the distribution of chromatin was in many instances irregular. Another characteristic of the altered cells was a pattern located paramembraneously in the cytoplasm, dependent on the plane of sectioning. It was analyzed partly on serial sections and partly on sections that were cut at right angles to each other. In sections cut parallel to the cutaneous surface, or close to it, the paramembraneous cytoplasm was characterized by an irregular, electron-dense pattern that had the appearance of a crown of thorns (figs. 1, 4, 9; *right upper corner* in figs. 11 and 13). If the sections were cut vertically, or approximately so, the pattern was different: In the cell periphery, fibrillar and/or filamentous elements formed chordlike designs of varying strength. Usually these were directed along the longitudinal axis of the cell [(*3*, *6*, *7*); figs. 7, 8, 10, 12, and 13]. In places these longitudinal bundles were in close contact with the intracytoplasmic fibrillar tufts that anchored to the cytoplasmic face of the desmosomes. They did not pass through the layered intercellular ultrastructures of the desmosomes. The same is true of the IFE of the normal mouse (*3*) and of epidermal hyperplasia provoked by Tween 60-type agents (*4*). In sagittal sections the bundles often appeared as huge formations, located in the poles of the cell, and reached the desmosomes within the intercellular bridge. Occasionally the bundles filled the entire bridge.

The *mitochondria* showed a variety of changes that were evident in sections cut in all directions. The number of mitochondria was decreased throughout and appeared scattered within the cytoplasmic matrix. The severity, type, and degree of alterations were more pronounced when treatment with carcinogen had been effective or prolonged. In the DMBA experiments, the changes were most marked and were apparent with low magnification (figs. 1, 4, 6 to 8, 10, 12, and 13). Figures 14 to 23 illustrate various types and stages of mitochondrial changes. A characteristic and frequent feature was an accumulation within the mitochondria of electron-

dense, round or globular, osminophilic inclusion bodies of hitherto unrecognized material. This pattern was observed so often that in the DMBA experiments only a few mitochondria appeared well preserved and contained the typical system of internal cristae (fig. 17; *right lower corner*). Within changed mitochondria, the internal cristae were arranged in a honeycomb-like manner (fig. 17), only remnants of the cristae were discernible (figs. 20 to 23), or the cristae were lacking (figs. 15, 16, and 19). Some of the mitochondria contained only 1 inclusion body, but usually 2 or 3 were seen in the same organelle. This pattern ("dégénérescence mitochondriale") resembled that of a spontaneous mouse sarcoma (*12*), but essentially deviated from that seen in uterine epithelium of estrous mice (*13*). In addition, these inclusion bodies totally deviated both in structure, size, and rate of occurrence from those sometimes, though extremely seldom, seen in Tween 60 series [*cf.* fig. 14 in (*4*)].

Stratum corneum.—This layer was irregular and much thicker than normal. The alterations were greater and more striking when the doses of carcinogens were high—the most severe changes were again in the DMBA experiments. A derangement of the lamellae was characteristic (fig. 3). Keratogenous material in various stages of maturation was present, and within the keratin matrix remnants of cellular organelles were distinguishable.

Experiment 2

A Single Local Application of 320, 160, 80, 60, 40, 20, and 10 γ of DMBA and 320 γ of MCA

Light and electron microscopic patterns were in accord. Even a single initial local exposure of the skin to DMBA and MCA brought about changes in the IFE. The degree of the alterations was directly correlated to the dose of the carcinogen: When a low dose was applied no deviations from the normal could be ascertained. The changes were most noticeable in the DMBA experiments: The great variability of changes in different places of the same treatment area was particularly apparent. A large amount of material was necessary to find them under the electron microscope. In structures in which the changes appeared most distinct, the changes were characterized by the same patterns that developed as a result of continuous exposure to the same carcinogens. Accordingly, ultrastructural alterations manifested themselves in cell surface-to-cell surface contact, cytoplasmic fiber formation, mitochondria, and in the stratum granulosum. Figures 24 to 26 show some of the effects of DMBA.

Cell surface-to-cell surface contact.—The intercellular spaces were large when the dose of carcinogen was high and the time from exposure to the carcinogen was short, *e.g.*, figure 24. In accordance with those under the light microscope, the spaces were larger in the basalmost layers. Intercellular spaces were clearly discernible on the 57th day (fig. 26). The basal cells, as well as the "compact basal-type" elements, were stretched toward their longitudinal axes. *Intercellular bridges* varied somewhat in

size, shape, and location. In places, adjacent cells were attached to each other and the direction of the bridges seemed to be toward the "lines of forces." In areas where the intercellular gaps were widest, the intercellular bridges often were lacking or ruptured, as in experiments with Tween 60-type agents (*4*). The ruptured bridgeheads had then drawn the *desmosomes* onto the cell surface. The ultrastructure of the latter appeared normal even though the amount of fibrillar tufts, anchoring to the internal cytoplasmic face of the desmosomes, was increased and electron-dense. The *dermoepidermal junction* and the *basal attachment devices* were of normal appearance.

Cytoplasm.—Figure 24 represents an intermediate electron microscopic pattern. In large areas the ultrastructure of the cytoplasmic matrix showed an almost normal pattern. However, when high doses (especially of DMBA) were administered, the paramembraneous cytoplasm of a few cells was contained in an electron-dense framework which formed a pattern resembling a crown of thorns, as in experiment 1. The *nucleo/cytoplasmic ratio* varied from normal to an increase of several degrees, even in the same section. Similarly, the *nucleo/cytoplasmic interface* varied from a scalloped to a relatively smooth surface. Occasional changes were seen within *mitochondria* that resembled those of experiment 1—usually when high doses of carcinogen were used. An accumulation of round or globular inclusion bodies was present. Mitochondrial alterations were seen in a small number, and, infrequently, when a long time had elapsed after exposure to the carcinogen. Changes could not be ascertained when low doses were used (*cf.* fig. 26, on the 57th-day level).

Stratum corneum.—The ultrastructure of this layer was not normal [*cf.* fig. 16 in (*3*)]. However, there were fewer changes than when the skin was exposed continuously to DMBA and MCA. Within the lamellae keratogenous material in various stages of maturation could be seen, as well as various cellular remnants (fig. 25).

Experiment 3

Continuous Local Treatment (6 and/or 12 Times a Week) With t. and s. Tween 60, After an Initial Local Application of 320 γ of MCA

The appearance under the electron microscope was in accord with that seen under the light microscope (*1*, *6–8*). Characteristically there was a greater variety of changes in different places of the treatment area than in experiment 2. It was possible to detect patterns of normal appearance (*3*), of a carcinogen effect (expts. 1 and 2), or of a Tween 60 effect (*4*). Electron microscopy revealed, even within the same cell, characteristics resembling both these effects. Figure 27 illustrates some patterns resulting from the two-stage treatment.

Cell surface-to-cell surface contact.—In figure 27 the *intercellular spaces* are extremely wide. In general, the spaces were wider in the basalmost layers of the hyperplastic IFE. The *intercellular bridges* showed considerable deviation in number and location: There were few, or part of

them had disappeared and those remaining were stretched. All these features were clearly discernible under the light microscope [*see* also figs. 16 and 17 in (*1*)]. Figure 27 reveals several *desmosomes* within the same intercellular bridge; this image may be due to the adjacent bridges. Most of the desmosomes are directed along the axes of the intercellular bridges. The intercellular layered ultrastructure of the desmosomal organelles was normal. The same was true of the ultrastructure of the *dermoepidermal junction* and *basal attachment devices*.

Cytoplasm.—The ultrastructure of the cytoplasmic matrix of nucleated cells in the hyperplastic IFE varied somewhat from one cell to another. Though 320 γ of MCA was used, only in a few instances, in the entire material, could features be distinguished which resembled those that developed as a result of continuous treatment with DMBA and MCA (expt. 1). In general, a substantial part of the cytoplasm was characterized by evenly scattered particulates. In this respect the pattern resembled that seen in the Tween 60 series [*cf.* figs. 13 and 14 in (*4*)]. Intracytoplasmic vacuoles, which in light and electron microscopy were typical of the Tween 60 effect, were not observed when exposure to MCA preceded treatment with the tumor promoter. In figure 27 a perinuclear framework is seen, to which adhere the central ends of stretched, chord-like filamentous tufts, the peripheral ends of which are anchored to the desmosomes. This pattern was most marked on the upper and lower poles of the cells. The *nucleo/cyctoplasmic ratio* varied somewhat from cell to cell. The *nuclei/cytoplasmic interfaces* showed a rather smooth course. The number of *mitochondria* varied; the vast majority were of normal ultrastructure. Only in a few cases were inclusion bodies seen.

Stratum corneum.—The degree of the changes varied within wide areas and sometimes had some features seen in mice treated with carcinogen; now and then the alterations were similar to those in the Tween 60 experiments.

Experiment 4

Continuous Local Treatment (6 and/or 12 Times a Week) With t. and s. Span 60, After an Initial Local Application of 320 γ of MCA

The electron microscopic pattern agreed with that seen with the light microscope. The makeup of the IFE essentially deviated from that in experiments 1 to 3 and was rather uniform, both in different places of the treatment area and in different animals. Figure 28 illustrates the effect.

Cell surface-to-cell surface contact.—There were no *intercellular spaces* between basal cells. The cell boundaries were in intimate contact with each other, and the folded cellular borders ran intimately parallel. As in normal IFE and experiments with Span 60-type agents (*4*), finger-like projections extended into the corresponding passages in the adjacent cells. Between basal cells no *intercellular bridges* were observed. The ultrastructure of *desmosomes*, *dermoepidermal junction*, and *basal attachment devices* was of normal pattern.

Cytoplasm.—The ultrastructure of the cytoplasmic matrix of basal cells had a relatively uniform pattern throughout: Some single cells deviated only slightly from normal. However, the image of the other basal cells remarkably resembled that developing as a result of exposure to Span 60 [*cf.* fig. 1 in (*4*)]. A relatively homogeneous scattering of densely located particles accumulated in the cytoplasm (fig. 28), occasionally condensed within the perinuclear halo. In some cells the particles formed a dense zone containing minute vesicular patterns, demarcated from the other cytoplasmic matrix. The *nucleo/cytoplasmic ratio* was perhaps higher than normal. The *nucleo/cytoplasmic interface* appeared accentuated and moderately scalloped. The ultrastructure of the *mitochondria* was normal.

Stratum corneum.—Parts of this layer appeared nearly normal and the electron microscopic pattern exhibited mild derangement.

Experiment 5

Two Benign Skin Papillomas Provoked by the Two-Stage Technique With t. Tween 60 and t. Tween 20 Used in Posttreatment

In light microscopy most skin tumors, promoted by dipole-type tumor promoters after a single initial local exposure to carcinogens, are fairly uniform, keratinizing, benign papillomas (*7*, *8*). The papillomatous epithelium contains, in principle, the same comparatively regularly formed cell layers as the surrounding hyperplastic nonpapillomatous IFE, though in somewhat different proportions. Next to the dermo-epidermal junction there are rather small, basal-type basophilic cells, arranged in several layers. Above them lie large, pale cells joined by intercellular bridges. The latter resemble IFE cells in differentiation. In more superficial layers the cell boundaries can no longer be distinguished. The keratinous mass is plentiful and often contains parakeratotic cells. Histoquantitative analyses have revealed that an approximate conformity exists between the percent distribution of basal-type cells and that of cells in differentiation, both in the papillomas and in the surrounding nontumorous hyperplastic IFE (*7*). The degree and quality of irregularity of the skin papillomas and that of the nontumorous IFE are directly correlated to the dose of the carcinogen given in pretreatment (*8*).

The electron microscopic image conformed to its light microscopic counterpart. Two patterns became particularly evident: (1) The ultrastructure of the nucleated cells closely resembled those in the surrounding hyperplastic IFE. (2) The nucleated cells were normal in appearance, with a good preservation of the pattern. Figures 29 to 32 illustrate some details in a benign papilloma promoted by t. Tween 20, a very weak tumor promoter. The pattern of the benign skin papilloma promoted by t. Tween 60 (a potent tumor promoter) did not deviate in any respect from that of t. Tween 20.

Cell surface-to-cell surface contact.—Figure 30 demonstrates the general appearance of the skin papilloma. *Intercellular spaces* occur between nucleated cells but are widest between basal-type cells. *Intercellular bridges* showed about the same course and pattern as seen with a dipole-type agent alone. The ultrastructure of the *desmosomes* (fig. 31), *dermo-epidermal junction*, and *basal attachment devices* bore a normal appearance.

Cytoplasm.—The ultrastructure of the cytoplasm (fig. 30) of the individual nucleated cells showed uniformity. The matrix resembled that seen in nontumorous hyperplastic IFE, and multilayered epithelium of other species. The *nucleo/cytoplasmic ratio* rarely deviated from normal. The *nucleo/cytoplasmic interface* exhibited only a slight variability from cell to cell and its course was rather normal. The ultrastructure of the *mitochondria*, including their internal cristae, was of normal pattern (fig. 32). Inclusion bodies, such as those observed in experiment 1, were not seen.

Superficial horny mass.—Characteristic of the benign skin papillomas provoked by the two-stage technique was, instead of the ordinary *stratum corneum*, the presence of masses of keratogenous, horny material—usually in enormous amounts. Figure 29 is an electron micrograph taken from this zone. The keratogenous mass contained remnants of epidermal cells and/or organelles.

DISCUSSION

The changes which develop in the IFE of the back of the mouse, as a result of local exposure of the skin to 7,12-dimethylbenz[*a*]anthracene and 3-methylcholanthrene, appear to have arisen *intra vitam* because: (*a*) The light and electron microscopic appearance can be compared, and related changes established by both techniques; (*b*) since the changes could be undeniably distinguished in light microscopy on conventional paraffin sections, it is not reasonable to consider the changes as being due to shortcomings in the electron microscopic technique; (*c*) the changes were qualitative, not quantitative only, and new formations developed; (*d*) the changes appeared regularly and in analogous experiments; (*e*) the changes were more pronounced when the skin was exposed to prolonged or effective doses of carcinogen; (*f*) there were large amounts of experimental material; and (*g*) experiments with carcinogens were repeated.

Of the ultrastructural alterations effected in the epidermis of the back of the mouse by local exposure to DMBA and MCA, the following were most characteristic: (*a*) In light microscopy various abnormalities in cell surface-to-cell surface contact indicate disturbances in detachment of epidermal cells ["stickiness" between parental and daughter cells (*2*)]; (*b*) development of new features, *e.g.*, a crown of thornlike patterns in the paramembraneous cytoplasm of the nucleated cells; (*c*) disturbances in keratinization; (*d*) accumulation of electron-dense inclusion bodies within mitochondria (figs. 1, 6 to 8, and 10 to 23)—the most characteristic and

regularly occurring alteration, and, as were other alterations, reproducible; and (*e*) high variability in degree and quality of the changes in different places of the treatment area. The changes were more pronounced, the more effective and/or more prolonged the exposure to carcinogens. It was not the question of a reversion of the epidermal cells to normal basal cells, to less-differentiated cell types, or of the cells remaining in the normal basal-cell stage, but rather it was the development of entirely *new cell types* in the IFE of this mouse strain. We have previously [*see* conclusions in (*3*, *4*)] considered it evident that in all hyperplastic conditions, in which the mouse IFE is changed into a distinctly multilayered structure and in which keratinization and desquamation have not appropriately occurred in due time, some kind of *delayed maturation of the epidermal daughter cells* must always be involved. In the experiments with carcinogens, the delay in maturation is real—*primary*. Furthermore, it must be *specific*. Histo-quantitative analyses have revealed that, despite a high-degree and progressively developing epidermal hyperplasia, the percentage of mitoses does not increase correspondingly, but this always happens in the regular hyperplasia caused by dipole-type promoters (*e.g.*, *1*, *7*). In carcinogen experiments, normal and pathologic mitotic figures are observed also in cells of the upper layers of the hyperplastic epidermis (*e.g.*, *1*).

The changes in the peripheral cytoplasm brought about by DMBA and MCA were related to those seen in low-power electron micrographs (*5*). The mitochondrial alterations resembled those in a spontaneous mouse sarcoma (*12*). It is known that the matrix of the normal mitochondria contains most of the enzymes of the citric acid cycle and that the enzymes of the respiratory cycle appear in the membraneous components. A fortunate laboratory could reveal the ultimate target(s) within the mitochondria of the carcinogenic effect.

When a single initial local application of DMBA and MCA preceded posttreatment with Tween 60, the electron microscopy revealed both the effect of the carcinogen and that of Tween 60 even within the same cell, a "competition" of the two noxae. A similar posttreatment with Span 60 partly masked the effect of the carcinogen.

As to the dipole-type *tumor promoters*, light and electron microscopic appearance were in accord (*1*, *4*). With locally applied *carcinogens*, light and electron microscopic appearance were also in agreement. In light microscopy, the hyperplastic response of the IFE caused by dipole-type tumor promoters was quite different from and opposite to that of locally applied carcinogens, DMBA and MCA. Accordingly, the same applies also to the electron microscopic patterns.

Most of the skin tumors provoked by the two-stage technique regress. It seems that two particular features are involved in this occurrence: (1) The effect of dipole-type tumor promoters is characterized by a substantial maintenance of the ultrastructure of the IFE cells. (2) A peculiar inherited feature of keratinizing tissues is the holocrine mode of function signifying that all the cell substance is shed. In normal IFE, the latter feature results in a gradual desquamation of the epidermal cells. In

benign, regularly formed skin papillomas both these characteristics are present. This might lead to a more or less complete disintegration of the papilloma cells that largely resemble those in IFE hyperplasia caused by dipole-type agents. The result is the regression of the tumor.

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PLATES

563711—00——29

The following designations on electron micrographs indicate cellular components: cb, cell boundaries; d, desmosomes; dej, dermoepidermal junction; ib, intercellular bridges; is, intercellular spaces; kg, keratohyaline granules; m, mitochondria; n, nuclei; t, tonofibrils; tf, tonofilaments.

PLATE 154

FIGURE 1.—Mouse #9126, male: 60 γ of DMBA, 6 times a week, 30th day. Electron micrograph of slightly tangential section from middle level of hyperplastic and severely deranged, polymorphic IFE. Pattern is characteristic of long-term exposure to DMBA. Nucleo/cytoplasmic ratio increased throughout. Nuclei show a scalloped nucleo/cytoplasmic interface. Paramembraneous cytoplasm of almost all cells characterized by irregular, electron-dense meshwork arranged like a crown of thorns (*arrow*). Majority of mitochondria contain electron-dense inclusion bodies. Intercellular spaces wide and uneven. Intercellular bridges irregular and interdigitating. These two patterns evident in light micrographs. Hair follicular cycle: telogen. × 8,000 [Presented previously in (*2*).]

FIGURE 2.—Mouse #20707, female: 60 γ of DMBA, 6 times a week, 30th day. Higher-powered electron micrograph from periphery of an altered cell. Intercellular spaces clear. Part of paramembraneous crown of thorns (*arrow*) is seen. A few mitochondria—one totally filled with electron-dense material. Hair follicular cycle: not definable. × 30,400

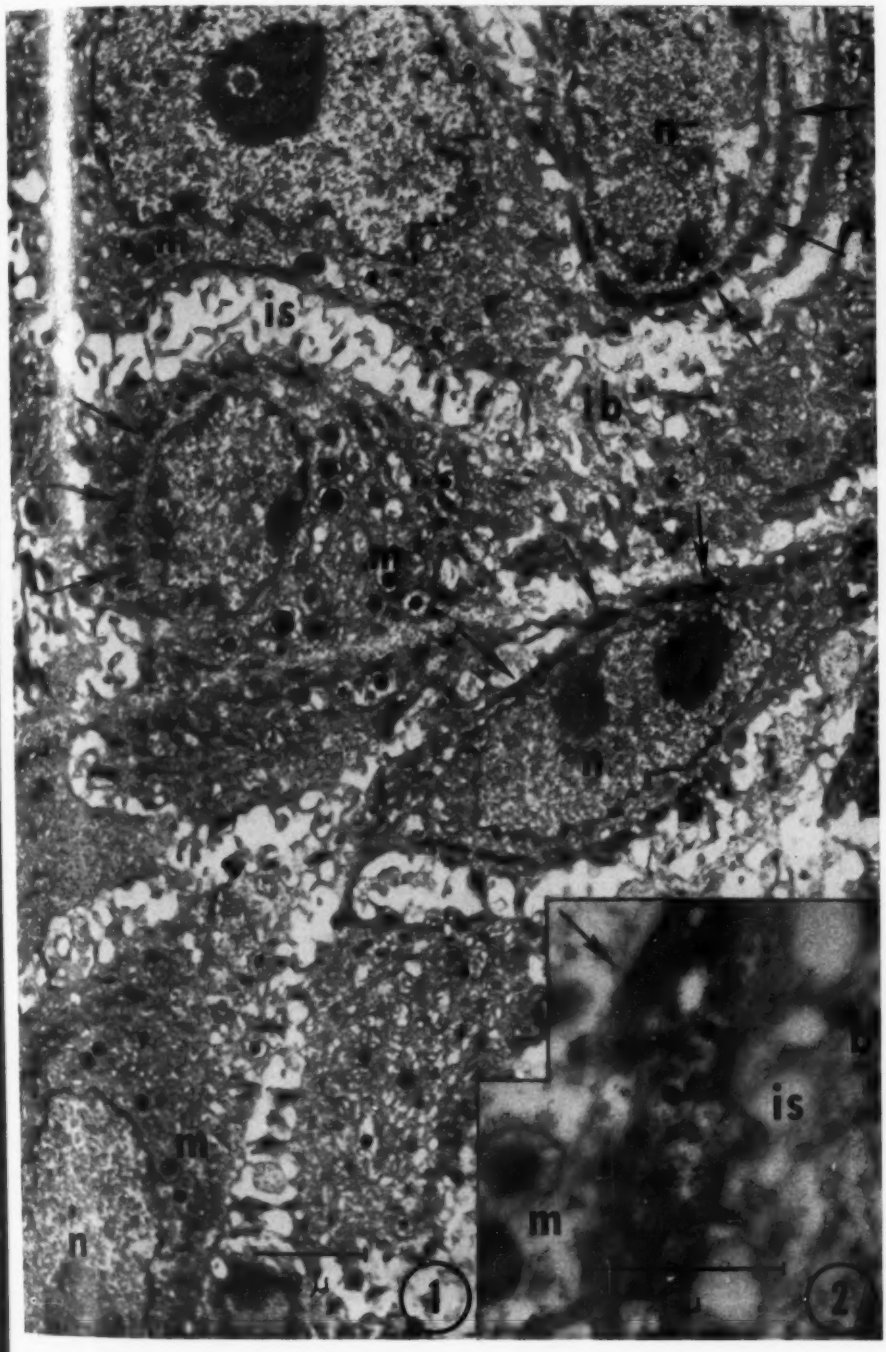
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PLATE 155

FIGURE 3.—Mouse #9122, male: 60 γ of DMBA, 6 times a week, 16th day. Electron micrograph from middle level of stratum corneum. Intermingling bundles of keratogeneous material in various stages of maturation. Hair follicular cycle: catagen. × 17,500

FIGURE 4.—Mouse #9126, male: 60 γ of DMBA, 6 times a week, 30th day. Electron micrograph of slightly oblique section from basal level of deranged IFE in same animal, but from another place than in figure 1. × 5,600

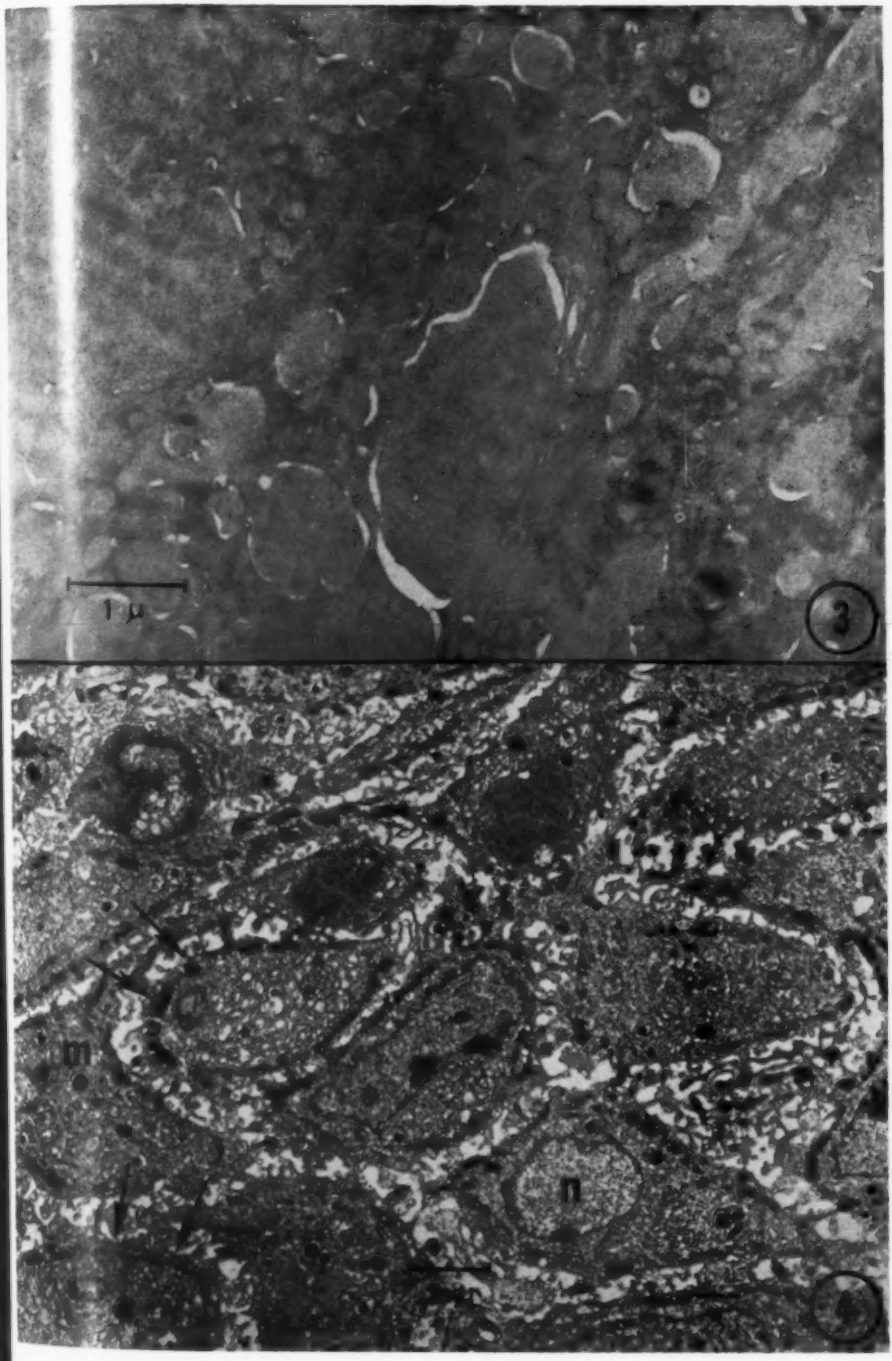
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PLATE 156

FIGURES 5 TO 8.—Mouse #20708, female: 60 γ of DMBA, 6 times a week, 30th day. Electron micrographs from sagittal sections of middle level of deranged IFE from *four different parts of same treatment area.* Patterns illustrate disturbed cellular contacts that appear as "stickiness" in light micrographs. These features are not encountered either in normal IFE or in hyperplasia brought about by Tween 60-type agents. In figure 5 *arrow* indicates a bundle of electron-dense fibrils and filaments that fill up a projection (? intercellular bridge) stretching between 2 adjacent cells. In figure 6 is seen a firm electron-dense bundle that joins 2 cells and seems to be in connection with tonofibrils at cytoplasmic periphery. Same pattern is particularly evident in figure 7. It also appears that these bundles are noticeable just at cell contacts. In sagittal sections, part of these bundles do not reach cell boundary. Figure 8 represents another type. Numerous mitochondria contain electron-dense inclusion bodies. Hair follicular cycle: not definable. Figure 5: × 7,800; figure 6: × 6,200; figure 7: × 5,900; figure 8: × 7,200

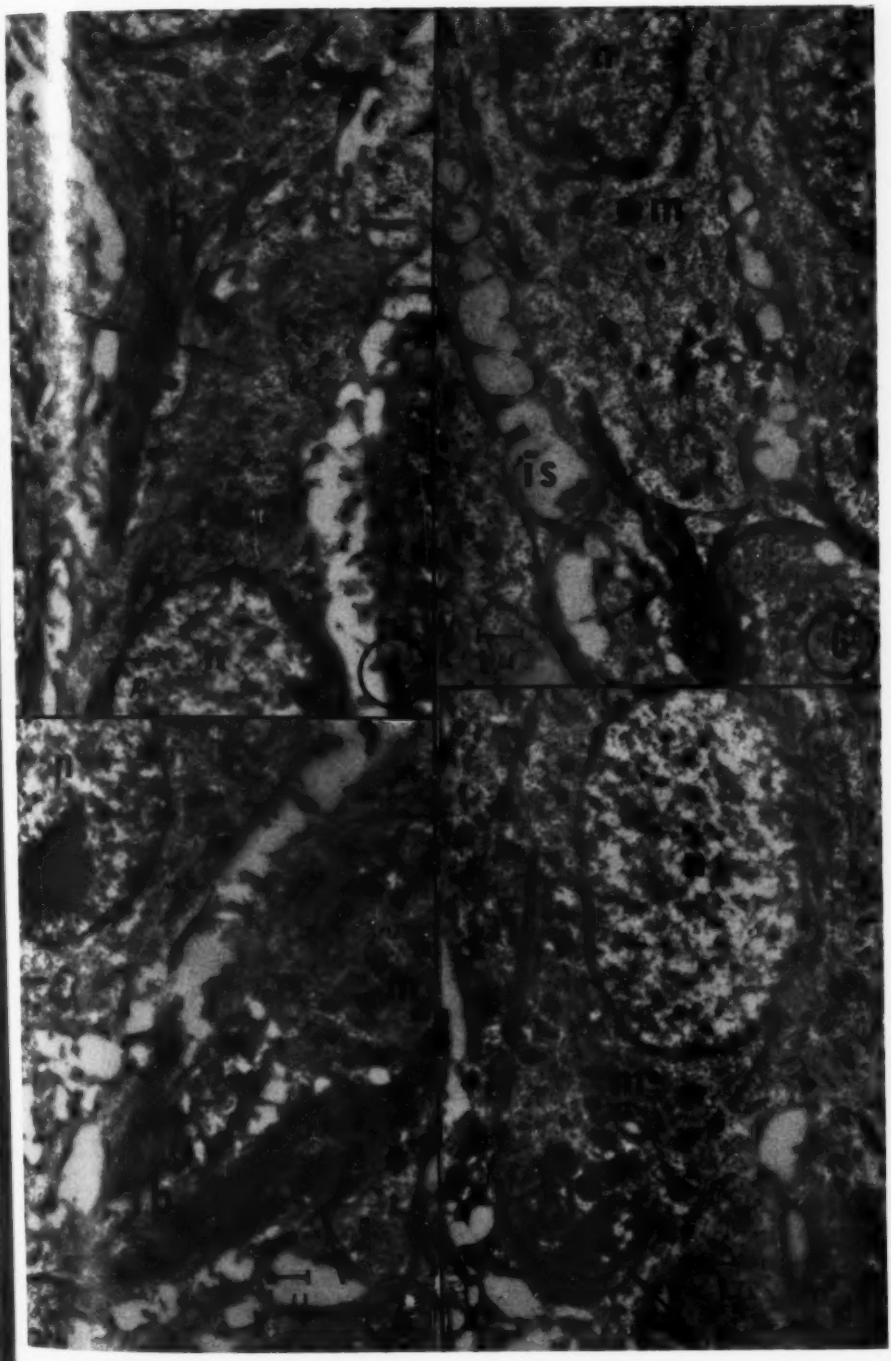
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PLATE 157

FIGURES 9 TO 12.—Mouse #20698, male: 60 γ of MCA, 6 times a week, 30th day. Hair follicular cycle: anagen (fig. 9), #20708, female: 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: not definable (fig. 10; same animal as in figs. 5 to 8), #20708: same animal as in figures 5 to 8, and 10 (fig. 11), and #20707, female, 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: not definable (fig. 12). Electron micrographs, from deranged IFE of *3 different animals*, illustrating variability of changes caused by MCA and DMBA. Figure 9 from a tangential section of middle level of basal cells. Enlarged nuclei almost fill up cells. Moderate intercellular spaces. Paramembraneous cytoplasm characterized by an electron-dense, irregular pattern that in this section plane appears like a crown of thorns—same phenomenon appears in figures 1 and 4. Figure 10 emanates from a slightly sagittal section from middle height of deranged, hyperplastic IFE. *Note* that now the paramembraneous pattern (*arrow*) appears more thread and bundle-like, which in places touches the desmosomal organelle. Figure 11, an obliquely sagittal section, shows a related pattern as figure 10. Fibrils and filaments situated closer to the perinuclear halo. Figure 12 a sagittal section from middle level of altered IFE: paramembraneous fibrils sparse. Some of mitochondria filled with electron-dense material. Figure 9: × 7,200; figures 10 and 11: × 6,900; figure 12: × 5,900

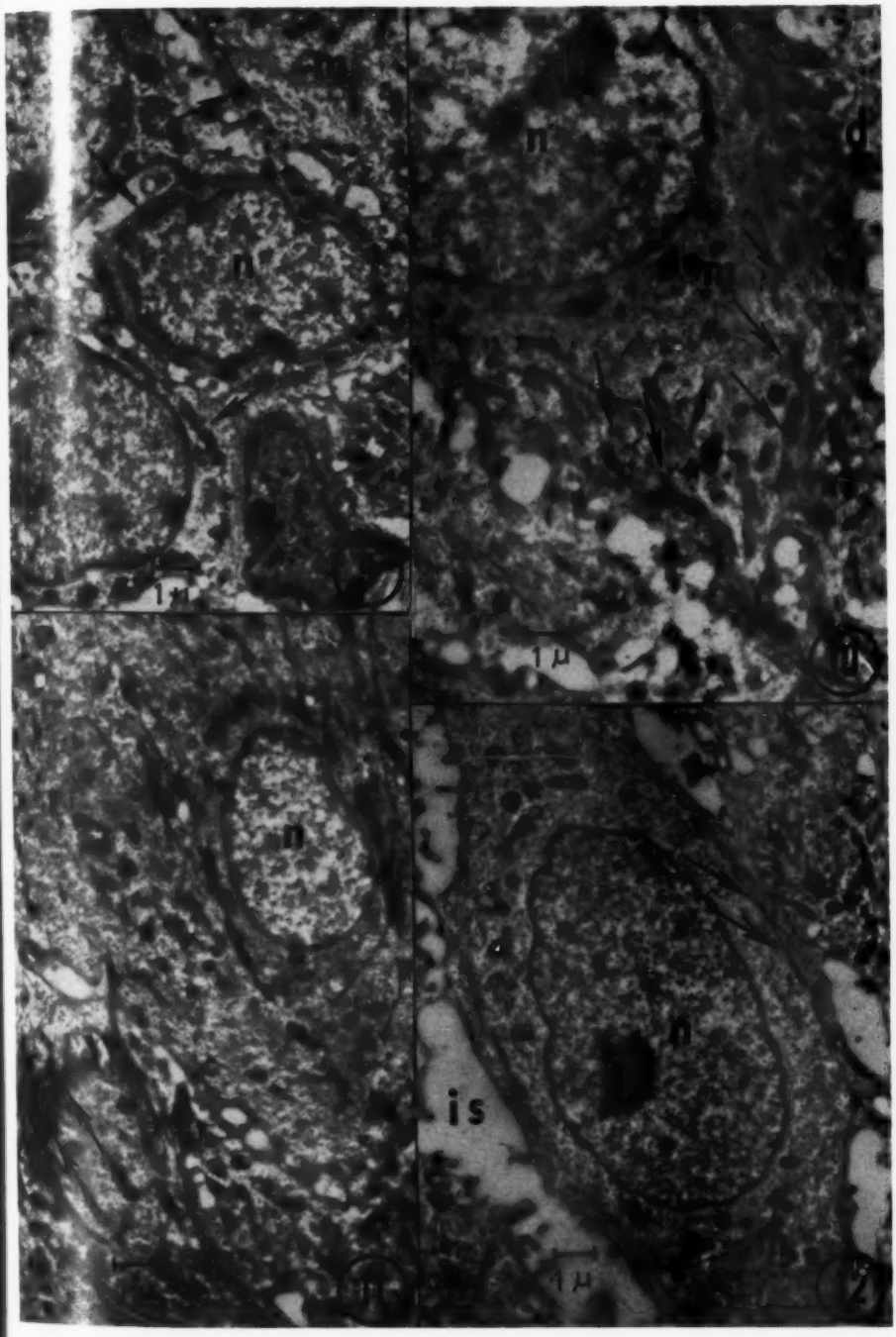
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PLATE 158

FIGURES 13 TO 17.—Mouse #20708, female: 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: not definable (fig. 13: same animal as in figs. 5 to 8, and 10 and 11), #20703, female, 60 γ of DMBA, 6 times a week, 16th day. Hair follicular cycle: not definable (fig. 14), #20707, female: 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: not definable (figs. 15 and 16; same animal as in figure 12), and #9126, male: 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: telogen (fig. 17; same animal as in figs. 1 and 4). Electron micrographs illustrating various types and stages of accumulation of inclusion bodies within mitochondria in deranged IFE of *4 different animals.* Figure 13 represents a slightly tangential section of upper layers. Almost all mitochondria are filled with osminophilic material. *Arrow* indicates paramembraneous network resembling that generally seen in tangential and/or oblique sections (*cf.* figs. 7, 8, 10, and 11). Figures 14 to 17 higher magnifications showing behavior of electron-dense inclusion bodies within mitochondria. Only a few mitochondria have typical internal cristae that cross the whole width of organelle; internal image of some of mitochondria shows a honeycomb-like arrangement. Figure 13: × 6,900; figure 14: × 29,000; figures 15 and 16: × 35,000; figure 17: × 36,000

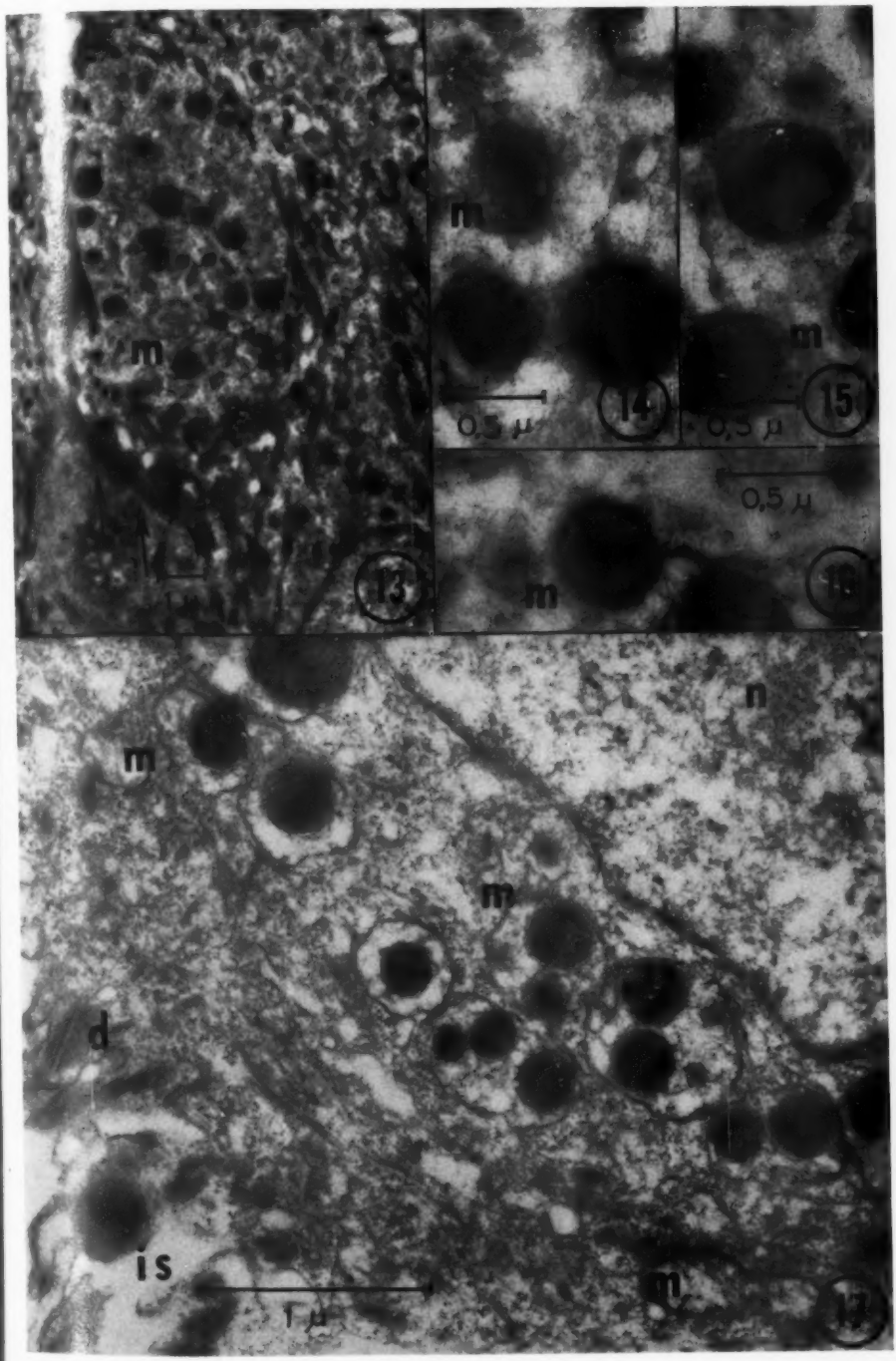
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PLATE 159

FIGURES 18 TO 23.—Mouse #20708, female: 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: not definable (fig. 18; same animal as in figs. 5 to 8, 10, 11, and 13), #20707, female: 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: not definable (fig. 19; same animal as in figs. 12, 15, and 16), #20718, male: 60 γ of DMBA, 6 times a week, 30th day. Hair follicular cycle: telogen (figs. 20 to 23). Electron micrographs illustrating further details of development of mitochondrial changes in *3 different animals*. Some mitochondria (figs. 20 and 23) still contain remnants of internal cristae. Pattern largely resembles the "dégénérescence mitochondriale" (*12*). Figure 18: × 15,500; figure 19: × 30,400; figures 20 to 23: × 73,400

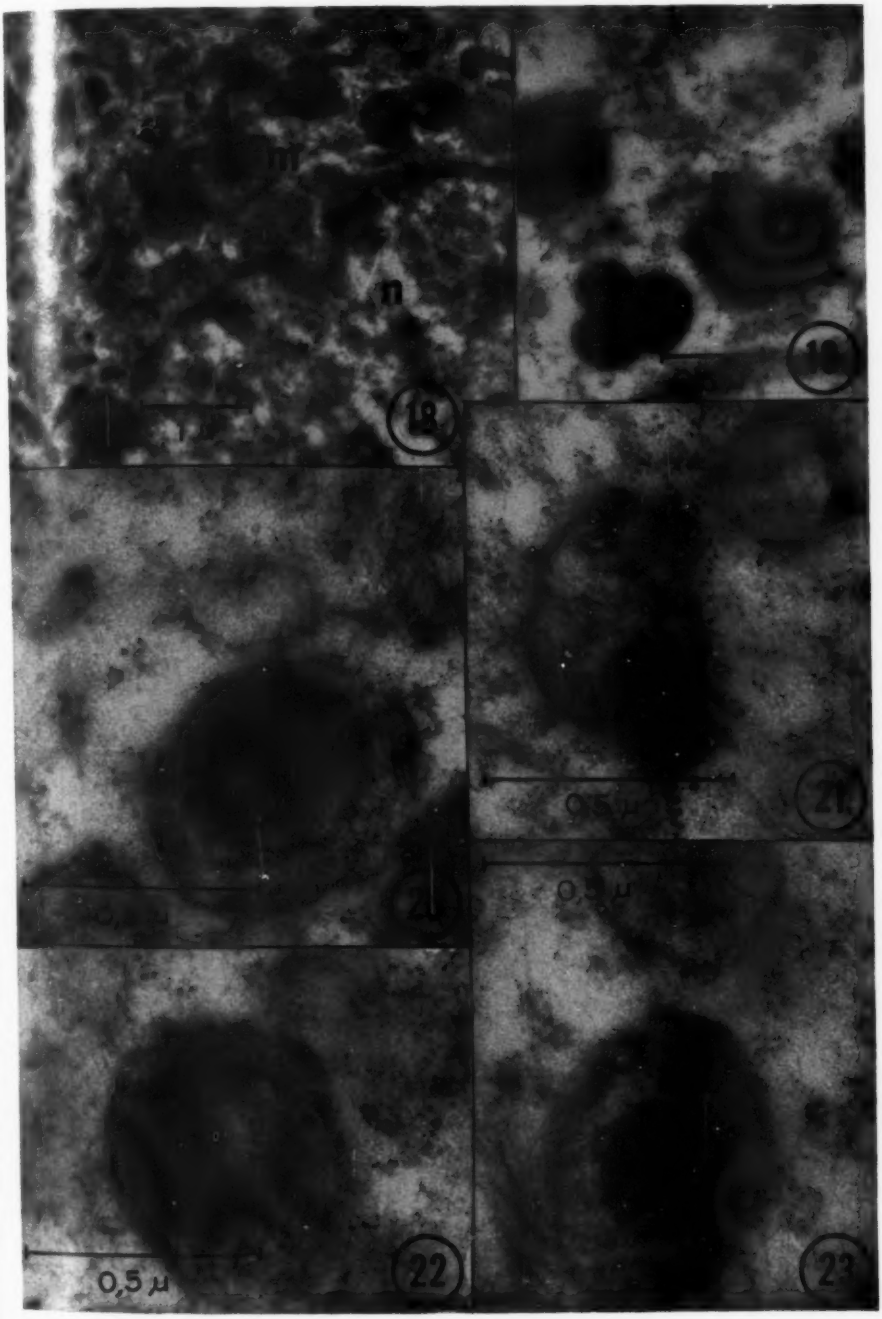
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PLATE 160

FIGURE 24.—Mouse #9716, male: single local application of 60 γ of DMBA without posttreatment, 2d day. Electron micrograph of parabasal and middle levels of altered and hyperplastic IFE. At parabasal level relatively wide intercellular spaces. Intercellular bridges somewhat irregular. Both are evident in light microscopy [*cf.* fig. 2 in (*1*)]. Cytoplasm rather finely granulated. Some mitochondria contain small inclusion bodies. Mitotic cell in *right lower corner*. Hair follicular cycle: telogen. × 7,100

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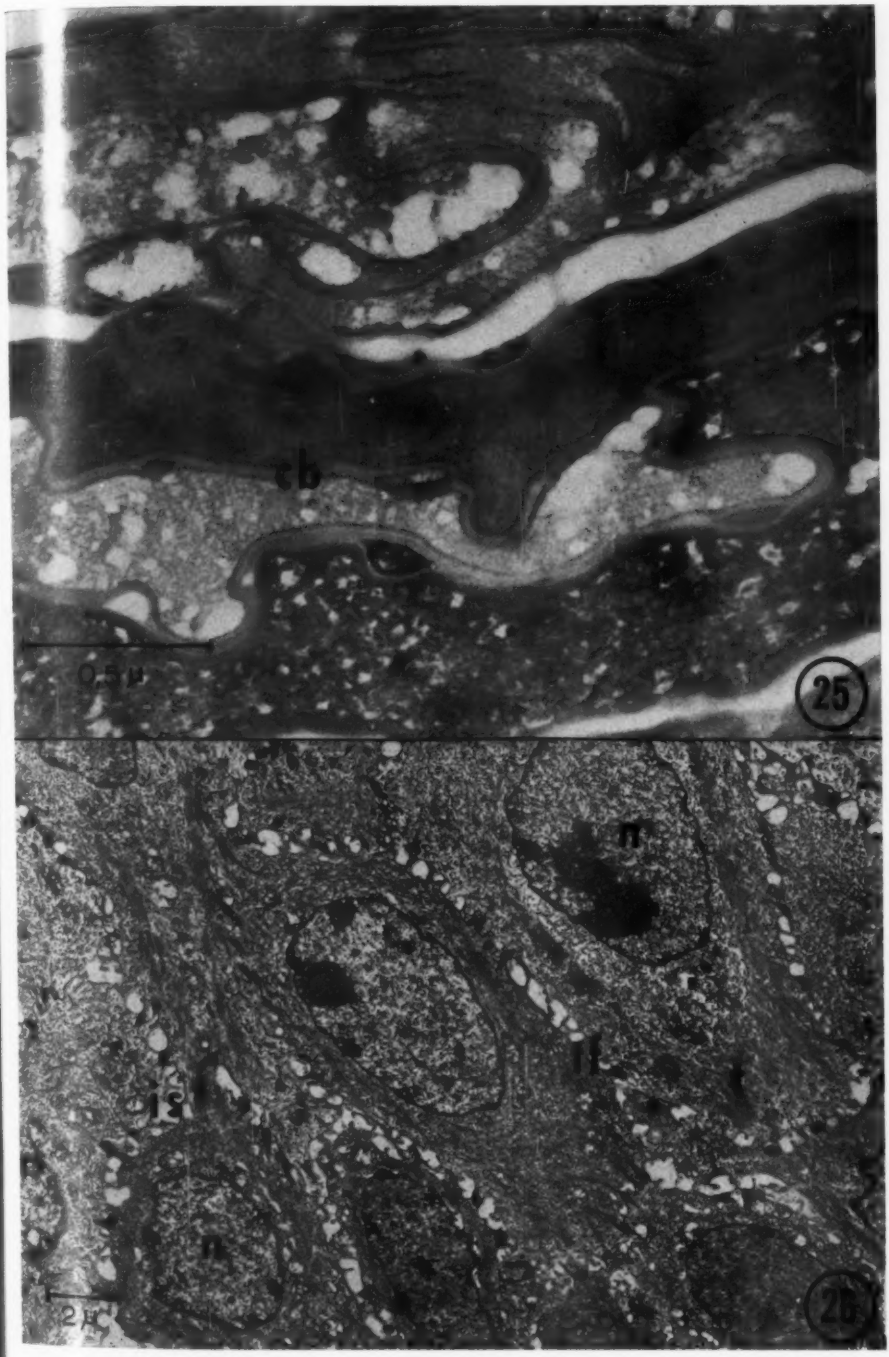
PLATE 161

FIGURE 25.—Mouse #7139, male: single local application of 60 γ of DMBA without posttreatment, 6th day. Electron micrograph, illustrating changes in stratum corneum. Disintegrating cells, probably in different stages of maturation, have approached cutaneous surface. Remnants of cell borders clearly distinguishable. Hair follicular cycle: telogen. × 60,000

FIGURE 26.—Mouse #20001, male: single local application of 60 γ of DMBA without posttreatment, 57th day. Electron micrograph of a somewhat oblique section. Compactness of cytoplasm did not depend solely on thickness of this section—in light microscopy same cells appear as basophilic "compactly built basal-type" cells [*see* description in (*1*, *6–8*)]. This is also true of distinct intercellular spaces. Nucleo/cytoplasmic ratio somewhat increased. Hair follicular cycle: between telogen and catagen. × 5,800



563711—60——30

PLATE 162

FIGURE 27.—Mouse #7375, male: s. Tween 60, 12 times a week, beginning 30 days after a single initial local application of 320 γ of MCA, 16th day. Electron micrograph from middle level of highly hyperplastic IFE. Image closely corresponds to light microscopic appearance [such as "stickiness" of adjacent cells, development of wide intercellular spaces; *see* fig. 16 in (*2*)]. *Arrow* shows chordlike bundles stretching between perinuclear fibrillar network and desmosomes. Cytoplasm finely granulated [*cf.* figs. 12 to 14 in (*4*)]. Hair follicular cycle: anagen. × 11,400

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PLATE 163

FIGURE 28.—Mouse #7400, male: s. Span 60, 6 times a week, beginning 30 days after a single initial local application of 320 γ of MCA, 16th day. Electron micrograph from basal portion of nonhyperplastic IFE. Pattern essentially differs from that developing as a result of continuous exposure to MCA and DMBA, and of post-treatment with Tween 60-type agents. On the contrary, it largely resembles that caused by Span 60-type agents alone. No actual intercellular spaces between basal cells. Cell borders intimately follow each other. Mitochondria of normal ultrastructure. Hair follicular cycle: anagen. × 18,400

FIGURE 29.—Mouse #16401, male: histologically benign skin papilloma promoted by t. Tween 20, 6 times a week, beginning 30 days after a single local initial application of 320 γ of MCA, 343d day. Electron micrograph from lower layers of the huge superficial keratin mass. × 16,000

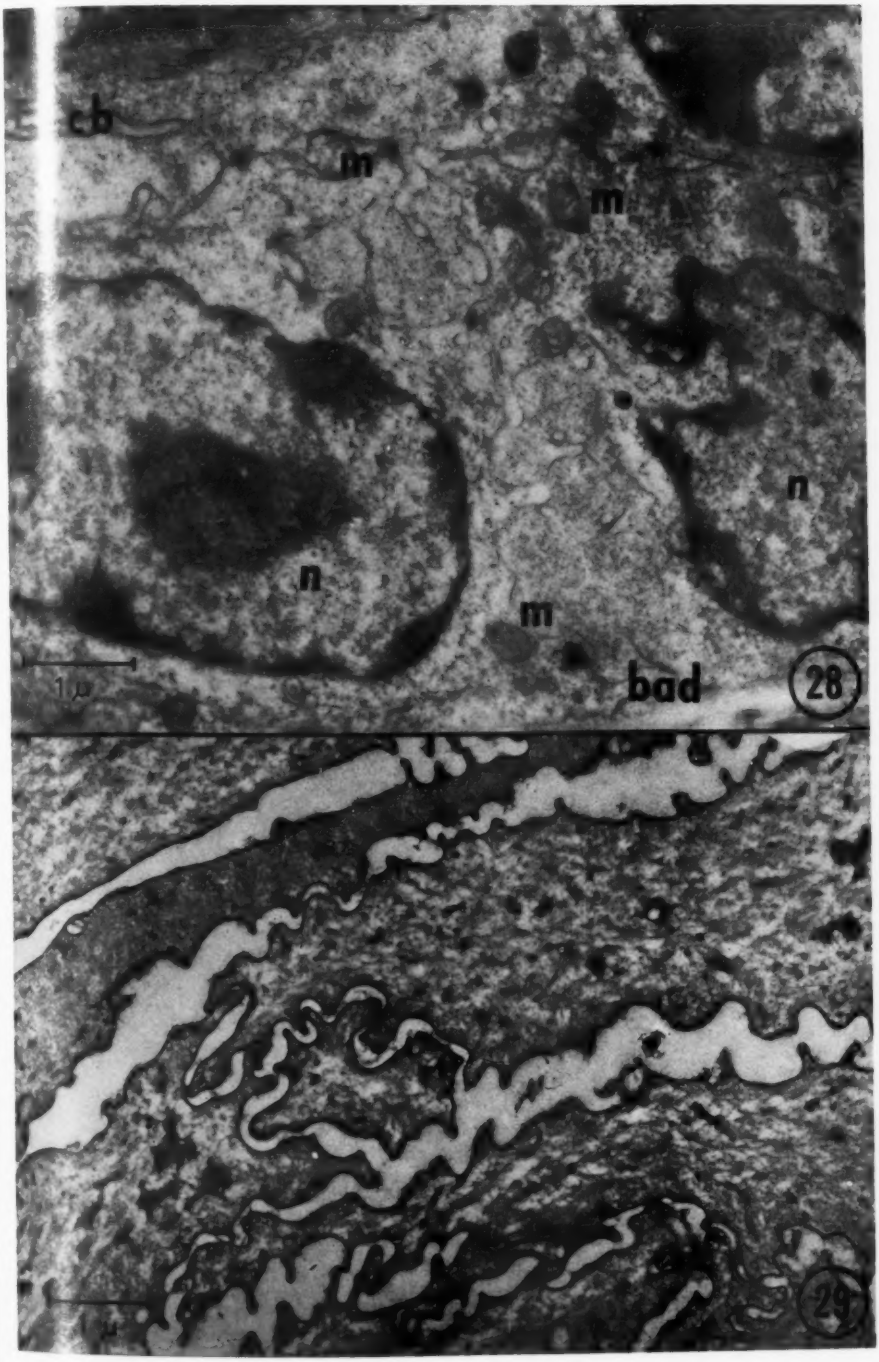
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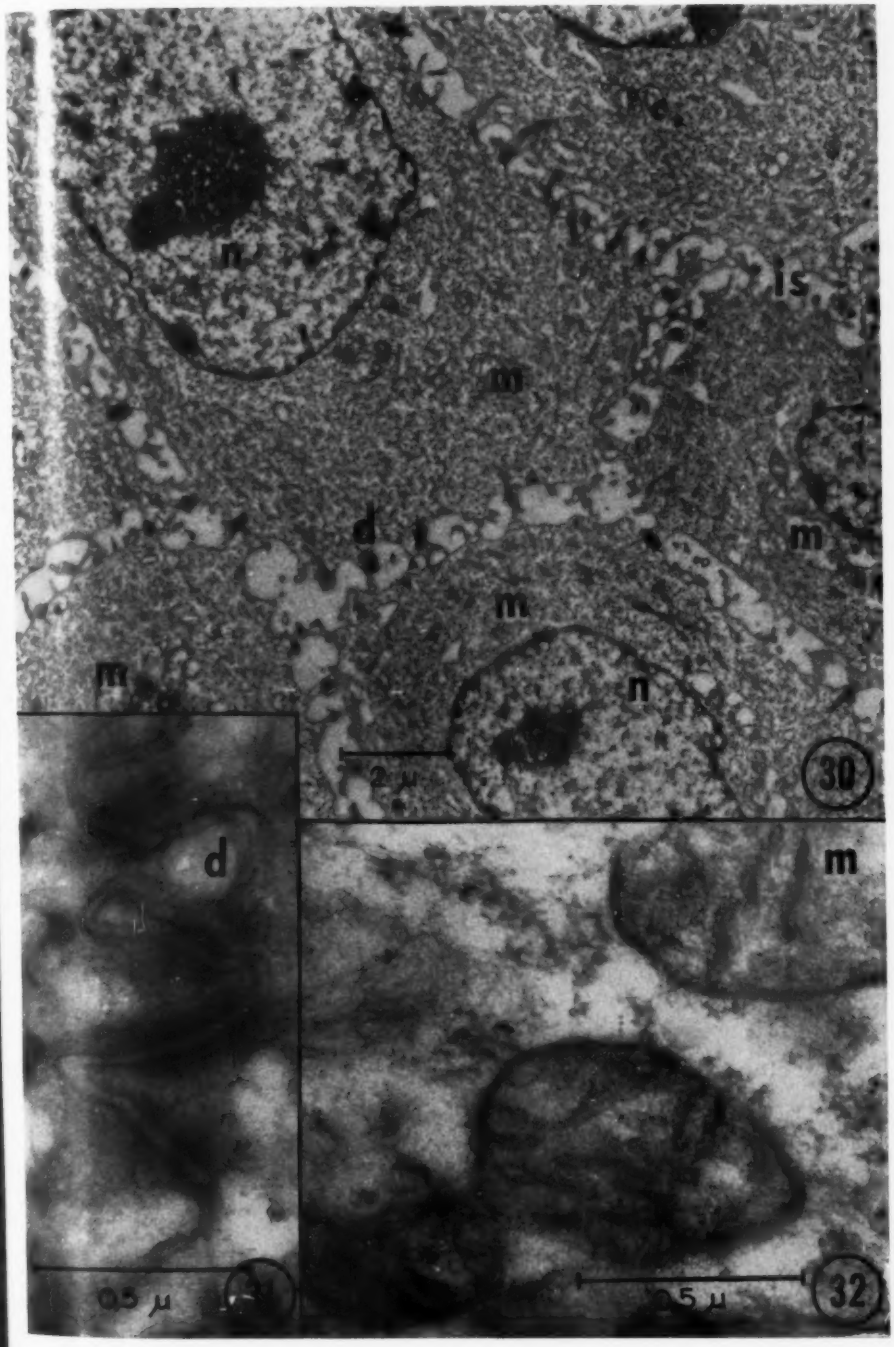
PLATE 164

FIGURES 30 TO 32.—Same animal as in figure 29: histologically benign skin papilloma. Electron micrographs illustrating about normal ultrastructure. Light and electron microscopy accord. Figure 30 is from middle portion of papilloma. Cytoplasm evenly granulated. *Note* scantiness of fiber formation. Intercellular spaces distinct. Figure 31 shows a row of desmosomes of normal pattern. Figure 32 represents some mitochondria of normal and characteristic appearance. Figure 30: × 8,100; figure 31: × 70,000; figure 32: × 67,000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Note on Results of Linder and Klein With Coisogenic Resistant Lines of Mice [1]

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IN STUDIES of mutations in transplantable tumors, George and Eva Klein and coworkers have been using four lines of mice, A/Sn, A.SW, A.BY, and A.CA, presumed to be coisogenic, which I sent them in March, 1955. Linder and Klein (*1*) have just reported the results of tests which prove rather conclusively that the lines are not only not coisogenic, but that lines A.SW, A.BY, and A.CA are not homozygous. Skin grafts made from strains A/Sn and A.SW to an F_2 generation derived from a cross between these strains gave ratios of susceptible to resistant animals, which indicated that at least 10 histocompatibility loci were segregating. Grafts within strains A.SW, A.BY, and A.CA, or to F_1 hybrids, of which one of these strains was a parent, often failed. This is indicative of a lack of homozygosity.

The series of crosses by which the coisogenic lines are produced (*2*) cannot give either complete coisogenicity or complete homozygosity. Nevertheless, the results reported by Linder and Klein are hardly compatible with well-established theoretical expectations. They are the more unexpected in view of the fact that Berrian and McKhann (*3*) have applied essentially identical tests to another coisogenic trio similarly produced (strains C57BL/10ScSn, B10.D2, and B10.LP) and found, in conformity with theoretical expectations, only single histocompatibility differences.

The unexpected outcome of Linder and Klein's tests with the three coisogenic strains on a strain-A background, and their contrast with the results of Berrian and McKhann with strains on a C57BL/10 background, suggested that there might have been some defect in the history of the coisogenic strains presumed to be on the former background. An examination of the records showed immediately that there was such a defect known to me at the time of its first detection in 1950 or 1951, but which I had subsequently forgotten.

The production of the first group of coisogenic resistant lines was started in 1946. These lines were destroyed in 1947 in the Bar Harbor fire. As soon as possible after the fire, we started new lines using, as

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the inbred parent, animals from various inbred strains sent to the Jackson Laboratory from other laboratories. Often there were a number of sublines of a given inbred strain to choose from. This was the case with strain A; here at least 5 sublines were available. The subline selected was A/L that had been kindly contributed by Eli Lilly and Company. The records do not show the reasons for the selection of this line; perhaps it was the first one available in sufficient quantity.

During the first stages of production of the lines, the transplantable tumors were not carried by my own assistant, but were obtained as needed from other members of the laboratory. This was unavoidable, since for several years after the fire animal space was severely limited. Because I did not carry the tumors, they were sometimes maintained in one subline of a particular inbred strain while another subline was used in the crosses to produce the coisogenic lines. Hence it was not until considerable progress had been made in the production of the lines on a strain-A background, probably in 1950, that I found that our strain-A tumor did not grow progressively in all mice of line A/L. I believe I am correct in saying that this was true not only of my subline of A/L, but also of another subline then maintained in the laboratory, and it has remained true of subline A/Ks, a Lilly subline, still maintained in the Jackson Laboratory. It was found at about the same time, as a result of outcrosses, that A/L was segregating for the gene *B*, normally not present in strain A. Again I believe that two sublines in the laboratory were implicated. Strain A/L also had a record of unusually high fertility that antedated its receipt at the Jackson Laboratory (information from Dr. Elizabeth Russell). All these facts point to contamination of strain A/L by an outcross, and a note to this effect appears on page 35 of *Mouse News Letter*, No. 5, July, 1951.

Because of the strong suspicion that A/L was not a typical A subline, and might not be homozygous, it was decided to replace it with a different subline. Beginning in June, 1951, animals of subline A/Wy were obtained to start a new breeding colony, but it was 1952 before they replaced A/L in the crosses used to produce the coisogenic lines. By this time the damage had been done. Line A.SW had progressed with particular rapidity—it was one of the first lines to be established—and was already in the first inbred generation. Lines A.BY and A.CA each had one mating to A/WySn before going into the inbred generations.

Although lines A.SW, A.BY, and A.CA must have been under considerable suspicion at this time, the extent of the damage was uncertain. Their successful use during the next 2 or 3 years in some tests for the identification of *H-2* alleles gave a sense of security, though we now know that because of the unique "strength" of the *H-2* locus, tests adapted for this purpose can be very unrevealing of other genetic differences. Nonetheless, when Dr. Klein wrote in 1955 to request some coisogenic strains, the bar sinister suspected in 1950–51 had been forgotten.

My own misconception in 1955 that strains A.SW, A.BY, and A.CA were on a normal A background led to a similar misconception on the

part of the Kleins. Because the experiments in which they used the lines turned out to hinge primarily on *H-2* differences, it was some time before the imperfections became a significant factor in this work and were suspected (*4*). I owe the Kleins a thousand apologies for having sent them, however unwittingly, lines with a defective history. It should, however, be made part of the record that these strains are not typical of the other coisogenic strains. The results of Berrian and McKhann (*3*) already cited should make this point clear.

REFERENCES

(*1*) LINDER, O., and KLEIN, E.: Skin and tumor grafting in coisogenic resistant lines of mice and their hybrids. J. Nat. Cancer Inst. 24: 707–720, 1960.

(*2*) SNELL, G. D.: Histocompatibility genes of the mouse. II. Production and analysis of isogenic resistant lines. J. Nat. Cancer Inst. 21: 843–877, 1958.

(*3*) BERRIAN, J. H., and MCKHANN, C. F.: Strength of histocompatibility genes. Ann. New York Acad. Sc. In press.

(*4*) KLEIN, G., and KLEIN, E.: Cytogenetics of experimental tumors. *In* Genetics and Cancer. Austin, Univ. Texas Press, 1959, pp. 241–270.

Announcements

Translation Program of American Institute of Biological Sciences

The American Institute of Biological Sciences is currently translating and publishing seven Russian research journals in biology: Doklady: Biological Sciences Section; Doklady: Botanical Sciences Section; Doklady: Biochemistry Section; Plant Physiology; Microbiology; Soviet Soil Science; and Entomological Review.

Five Russian monographs have been published: A. L. Takhtajan: Origins of Angiospermous Plants; G. F. Gauze: Problems in the Classification of Antagonists of Actinomycetes; A. A. Zakhvatkin: Arachnoidea; B. I. Pomerantzev: Arachnida; B. N. Nikitin, editor: Marine Biology, Trudi Institute of Oceanology, Vol. XX. The manuscript of L. P. Breslavets, Plants and X Rays, is in press.

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Manuscripts Approved for JNCI, September 15 to October 15, 1960

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"The distribution of cancer mortality between ethnic subgroups of the white population of New York City, 1953–58," by Vaun A. Newill.

"Studies of some antigens of the L strain mouse fibroblast," by Joseph H. Kite, Jr., and Donald J. Merchant.

"Survival of patients with cancer of the large intestine and rectum, Connecticut, 1935–54," by Fred Ederer, Sidney J. Cutler, Henry Eisenberg, and John R. Keogh.

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"Tumor induction by genetically homogeneous lines of polyoma virus," by Ernest Winocour and Leo Sachs.

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